Department for Farm Animals
Clinic of Reproductive Medicine
of the Vetsuisse-Faculty University of Zurich

Director: Prof. Dr. med. vet. Heinrich Bollwein

Work under the academic supervision of
Prof. Dr. med. vet. Heinrich Bollwein
PD Dr. rer. nat. Stefan Bauersachs

Transcriptomics of the endometrium in mares susceptible and resistant to persistent breeding-induced endometritis using RNA-Sequencing

Inaugural Thesis

to obtain the title of Doctor from the

Vetsuisse Faculty University of Zurich

submitted by

Amr Salah Mohamed ELSHALOFY

Veterinarian

of Damietta, EGYPT

Approved at the request of

Prof. Dr. med. vet. Heinrich Bollwein, Supervisor

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Vetsuisse Faculty, University of Zurich (2020)

Amr Salah Mohamed ELSHALOFY

Clinic of Reproductive Medicine, Secretary of Institution: mwunder@vetclinics.uzh.ch

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Abstract

This study aimed to investigate the susceptibility of mares to persistent breeding induced endometritis (PBIE) and fertility by transcriptomic analysis of uterine samples using RNA-Sequencing. Cytobrush samples were collected from the uterus of 81 broodmares before artificial insemination (AI). Susceptibility to PBIE was evaluated by the amount of intrauterine fluid 24 hours after AI and fertility was determined by a sonographic pregnancy diagnosis two weeks after ovulation. RNA expression profiles were compared between susceptible non- pregnant (SNP) mares (n=16) and resistant pregnant (RP) mares (n=21) as well as between susceptible pregnant (SP) mares (n=9) and susceptible non-pregnant (SNP) mares (n=16). Sixty-six genes were differentially expressed (DEGs) between SNP- and RP mares and 60 DEGs were noticed between SP- and SNP mares. In SNP compared to RP mares, gene expressions regulating steroid hormone metabolism and chemotaxis of neutrophils were lower, while those participating in uterine inflammation were higher. DEGs related to extracellular matrix degradation, tissue adhesions and fibrosis were lower in SP mares than in SNP mares, while gene expressions, which are related to uterine cell proliferation, differentiation, and angiogenesis were higher in SP mares than in SNP mares. In conclusion, there were substantial differences in the uterine transcriptome profiles according to susceptibility to persistent breeding induced endometritis and fertility in mares.

KEYWORDS: equine, persistent breeding induced endometritis, fertility, RNA-Seq, cytobrush.

Zusammenfassung

In dieser Studie wurde die Anfälligkeit von Stuten für eine persistierende, besamungsinduzierte Endometritis (PBIE) und die Fertilität anhand der Transkriptomanalyse uteriner Proben mittels RNA-Sequenzierung (RNA-Seq) untersucht. Dazu wurden von 81 Stuten vor der künstlichen Befruchtung (KB) Zytobrush-Proben vom Uterus entnommen und eine PBIE anhand der Menge intrauteriner Flüssigkeit 24 Stunden nach der KB sowie die Fertilität 2 Wochen später mittels Ultraschall diagnostiziert. Die RNA-Expressionsprofile wurden zwischen anfälligen nicht-trächtigen (ANT; n = 16) und resistenten trächtigen Stuten (RT; n = 21) sowie zwischen AT (n = 9) und ANT (n = 9) verglichen. Zwischen ANT und RT waren 66 Gene unterschiedlich exprimiert und zwischen AT und ANT 60 Gene. Bei ANT waren im Vergleich zu RT die Expressionen von Genen, die den Steroidhormonstoffwechsel und die Chemotaxis von Neutrophilen regulieren, geringer, und von Genen, die an einer Uterusentzündung beteiligt sind, höher exprimiert. Gene, die mit dem Abbau der extrazellulären Matrix, Gewebeadhäsionen und Fibrosierungen zusammenhängen, waren bei AT niedriger als bei ANT exprimiert, während Gene, die mit der Proliferation und Differenzierung uteriner Zellen und der Angiogenese zusammenhängen, bei AT höher waren exprimiert waren als bei ANT. Zusammenfassend unterschieden sich die Transkriptom-Profile in Abhängigkeit von der Anfälligkeit gegenüber einer besamungs-induzierten Endometritis und der Fertilität der Stuten.

SCHLÜSSELWÖRTER: Pferd, persistierende besamungsinduzierte Endometritis, Fruchtbarkeit, RNA-Seq, Zytobrush.

Abbreviations

ADAMTS	A disintegrin and metalloprotease with a thrombospondin motif
ADAMTS6	ADAM metallopeptidase with thrombospondin type 1 motif 6
AI	Artificial insemination
APOE	Apolipoprotein E
BIE	Breeding induced endometritis
CD44	Cluster domain 44
cm	Centimeter
COL4A3	Collagen type IV alpha 3 chains
COL5A2	Collagen type V alpha 2 chains
CPM	Counts per million
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C chemokine receptor
DEGs	Differentially expressed genes
E2	Estrogen
EBD1	Equine β-defensin 1
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FBN1	Fibrillin 1
FDR	False discovery rate
FGF9	Fibroblast growth factor 9
FGFR	Fibroblast growth factor receptor
FN1	Fibronectin
GDF10	Growth differentiation factor 10

h	Hour/ Hours
HA	Hyaluronic acid
HB-EGF	Heparin-binding EGF-like growth factor
HPF	High power field
IL 1	Interleukin 1
IL 17	Interleukin 17
IL 6	Interleukin 6
IL23A	Interleukin-23 subunit alpha
ITGB3	Integrin beta 3
KDR	kinase insert domain receptor
LDLR	low-density lipoprotein receptor
LPS	Lipopolysaccharide
LYZ	Lysozyme
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
NCBI	National center for biotechnology information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOS-2	Nitric oxide synthase 2
OPN	Osteopontin
P4	Progesterone
PBIE	Persistent breeding induced endometritis
PMNs	Polymorphonuclear neutrophils
RIN	RNA integrity numbers
RNA	Ribonucleic acid

RNA-Seq	RNA-sequencing
RNF175	Ring finger protein 175
RNP	Resistant non pregnant
ROBO 2	Roundabout 2
RP	Resistant pregnant
SLPI	Secretory leukoprotease inhibitor
SNP	Susceptible non-pregnant
SP	Susceptible pregnant
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
Th17	T helper 17
TNF-alpha	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
αIIbβ3	Alpha-IIb / beta-3

1. Introduction

Endometritis has been ranked as the most important reason for subfertility in mares and the third most frequent disease of adult horses, reflecting a substantial economic and genetic loss in the equine breeding sector (Traub-Dargatz et al. 1991, Gutjahr et al. 2000). LeBlanc (2010) categorized the inflammation of the endometrium into acute/ chronic infectious, persistent breeding induced (PBIE), and degenerative endometritis. Most common are bacterial infections and PBIE (Christoffersen and Troedsson 2017).

Transient breeding-induced endometritis (BIE) is a local, physiological inflammatory reaction of the equine uterus that begins shortly after breeding to eliminate invasive microorganisms and excessive seminal components (Troedsson 1999). While a timely regulated inflammatory response is desirable to establish a healthy uterine environment before embryo descent, the persistence of this condition (PBIE) may develop an embryotoxic uterine environment in addition to triggering a premature luteolysis, which subsequently threatens the maintenance of pregnancy (Causey 2006, Woodward and Troedsson 2013a). Mares are classified as resistant or susceptible to PBIE based on their ability to clear uterine inflammation and fluids in a timely post-breeding manner. Many mares (85-90 %) are reproductively healthy and can efficiently control BIE within 24 hours of breeding, while a minority (10-15 %) is unable to regulate this inflammatory reaction on time, so they are considered susceptible to PBIE (Troedsson, 1999, Christoffersen and Troedsson, 2017).

PBIE has a multi-factorial nature that makes its diagnosis challenging. The prediction of susceptibility is usually limited to the history of subfertility/ abnormal intrauterine fluid accumulation or the presence of score IIB-III endometrium according to the histological classification of Kenney and Doig, so being able to detect the susceptible mares in advance is critical to provide them with a special breeding management to increase pregnancy rates (Kenney and Doig 1986, Troedsson et al. 1993, Maischberger 2008, Rasmussen et al. 2015).

Although swab, cytobrush, low-volume lavage, and biopsy are used to gather uterine samples for the classical diagnosis (microbiology, cytology, and histology) of endometritis in mares (Ferris 2016), the histological evaluation of uterine

biopsy samples has been considered to be the most specific and sensitive diagnostic method for endometritis particularly for deep chronic uterine infections (De Amorim et al. 2015).

Since a few years, the uterine biopsy is not only used for histological assessment but also for investigation of the expression of some genes in the equine uterus. Recently, the RNA-Seq technique has been used to provide a broad range of transcriptomic data that helps to understand genetic changes and biological dynamics associated with physiological and pathological processes in different tissues of organs (Metzker 2010, Ozsolak and Milos 2011). The uterine RNA-Seq allows a better understanding of the pathophysiology of endometritis and provides additional, more objective information about the susceptibility to PBIE compared to subjective histological evaluations (Woodward and Troedsson, 2015, Christoffersen and Troedsson, 2017).

Cytobrush can collect cells of high integrity from both the surficial and deep layers of the uterus that may be used for microbiological, cytological as well as transcriptomic assessments (Cocchia et al. 2012, Ferris et al. 2016, Palma-Vera et al. 2015). Equine breeders and practitioners prefer using the cytobrush to diagnose endometritis rather than biopsy under field conditions as cytobrush is considered an effective, practical and non-invasive technique (Overbeck et al. 2011, Buczkowska et al. 2014). RNA-Seq of the uterus in equine and human medicine was up to now performed on biopsy samples (Woodward and Troedsson, 2015), while in cows and recently in mares it was shown that cytobrush samples can provide an adequate quantity and quality of RNA isolates for transcriptomic analysis (Minten et al. 2013, Wagener et al. 2017, Weber et al 2020). Cytobrush samples have been reported to show higher levels of gene expression than biopsy samples in cows with endometritis (Fagundes et al. 2019).

However, previous studies were focusing on the retrospective diagnosis of PBIE by investigating some genes in biopsy samples after challenging the uterus by insemination or inoculation of bacteria (Woodward and Troedsson, 2015), only one study has tried to investigate the susceptibility of mares to develop a PBIE by examining the association between the endometrial histology score and intrauterine fluid accumulation post-breeding (Woodward et al. 2012).

Recently, Marth et al. (2018) suggested that equine β-defensin 1 (EBD1), lysozyme (LYZ), and secretory leukoprotease inhibitor (SLPI) could be used as diagnostic markers to identify PBIE-susceptible mares with 94% overall sensitivities and specificities. In the last- mentioned study, the uterine biopsy samples were collected in estrus and diestrus for transcriptomic analysis of selected immune genes from mares that were retrospectively categorized as susceptible or resistant to PBIE during previous estrous cycles, the authors did not correlate their findings of uterine gene expression with the intrauterine fluid accumulation post-breeding or fertility results.

We hypothesized that mares susceptible and resistant to PBIE may have different uterine RNA profiles even before they are challenged by breeding. Furthermore, the uterine RNA profiles before breeding may affect the fertility of the mares which may reveal some molecular biomarkers for the susceptibility to PBIE as well as mare fertility. Our study aimed to examine the whole transcriptome profiles of uterine cytobrush samples collected before breeding from an uncategorized broodmare population and subsequently to compare the mRNA expression levels based on the post-breeding uterine reaction and the result of the pregnancy diagnosis in order to investigate 1) the susceptibility to PBIE by comparing the uterine transcriptome profiles in susceptible mares that did not become pregnant in comparison to resistant mares that became pregnant, as well as to investigate 2) the ability of susceptible mares to become pregnant with the proper breeding management by comparing the uterine transcriptome profiles of susceptible that became pregnant and susceptible mares that did not.

2. Materials and Methods

2.1 Mares

The current study was performed from May to August 2019 on Standardbred mares at a large private equine stud farm in Mecklenburg-Vorpommern, Germany. Mares were light horses (400-550 kg) kept on straw-bedded boxes with permanent access to hay, grain, and water, and were turned out daily to pasture. We selected mares that were ≥10 years of age with no history of reproductive disorders or clinical symptoms of endometritis during previous estrous cycles and were planned to be artificially inseminated (AI) with cooled semen from an already proven fertile stallions (certified for the commercial breeding purpose)

2.2 Study design

The schedule for reproductive management and sampling of the mares is shown in Fig. 1. After a routinely performed transrectal palpation and ultrasonography of the genital tract to evaluate their ovarian and uterine status, uterine cytobrush samples were collected before AI from 81 mares during estrus. Afterwards, ovulation was hormonally induced. Twenty-four hours later mares were inseminated with cooled semen and further 24 hours mares were examined for the onset of ovulation (mares which did not ovulate within 48 hours of induction were excluded from the study). As 9 mares did not ovulate within 48 hours after induction of ovulation, the remaining 72 mares were grouped based on their maximum diameter of intrauterine fluid. Mares showing intrauterine fluid ≥ 2cm in diameter 24 hours after AI were defined as susceptible mares (n=25) while mares with 0 -< 2 cm intrauterine fluid were called resistant mares (n=47). Mares were further subdivided according to the result of pregnancy check into 4 groups: susceptible non-pregnant (SNP, n=16), susceptible pregnant (SP, n=9), resistant non-pregnant (RNP, n=26), and resistant pregnant (RP, n=21) mares (Fig. 2).

2.3 Collection of uterine samples

Two double-guarded uterine cytobrushes (Minitüb GmbH, Landshut, Germany) per mare were collected. The first cytobrush sample was used for the extraction of total RNA while the second one was used for cytological assessment. All samples were collected at mid-estrus (1-3 days before AI) when the mares had a dominant follicle (mean diameter of about 35 mm), a moderate endometrial edema, and a relaxed cervix. Samples were collected according to Dascanio and Ferris (2014) with some modifications. Briefly, mares were prepared as for AI. After removing feces from the rectum, the tail was wrapped and tied up. The perineal region was washed with non-residual soap and rinsed with fresh water at least three times to remove any obvious debris. Then, the area was dried using disposable paper towels. The veterinarian wore a clean, inverted rectal sleeve with sterile gloves on top. Sterile lubricant was put on the knuckles, thumb, and dorsal wrist area. The tip of double-guarded cytobrush was held and covered in the palm. With a slight rotating motion, the hand was passed into the vagina until the external cervical os was located, the index finger was introduced gently into the cervical canal,

followed by the cytobrush.After passing the cervical canal, the brush itself advanced into the outer, then the inner guards, to be in contact with the endometrium of the uterine body. Then, it was gently rolled for approximately 15 seconds and retracted together with the inner guard from the outer guard, the outer guard was left in the mare until collecting the second sample to avoid additional manipulation with the cytobrush. The first cytobrush was directly inserted into a 1.5 ml reaction tube with 350 µl of lysis buffer RNeasy micro kit (Qiagen, Hilden, Germany), rolled for 20 seconds. The reaction tubes were snap-frozen and stored in a liquid nitrogen tank until further analysis.

A second new cytobrush with an inner guard only was inserted through the previous outer guard into the uterus. After collection of the sample, the brush was retracted back to the inner guard, then to the outer guard. and finally, the device was removed from the mare. The cytobrush was rolled on two microscope slides, air-dried, fixed, and stained with Diff-Quick for cytological evaluation.

2.4 Breeding management

Using a standard gynecological procedure described by McCue (2014), the reproductive tract of the mares was examined using transrectal ultrasonography (Aloka Prosound 2, Hitachi, Japan) to determine the optimal time for uterine sampling and AI as well as to confirm the absence of any clinical symptoms of endometritis (i.e. an abnormal pattern of uterine edema in relation to follicular size, intrauterine fluid accumulation with a diameter ≥ 2 cm before or 24 hours after breeding, echogenic intra-uterine particles). The ovulation was induced, if the mares showed a preovulatory follicle ≥35 mm, moderate endometrial edema and a relaxed cervix by an intramuscular injection of 2500 IU human Chorionic Gonadotropin (Ovogest ® MSD Animal Health, Unterschleißheim, Germany). The mares were inseminated 24 h later using approximately 10-20 ml of cooled semen with a progressive motility > 50 % and > 300 million sperm/dose of insemination.

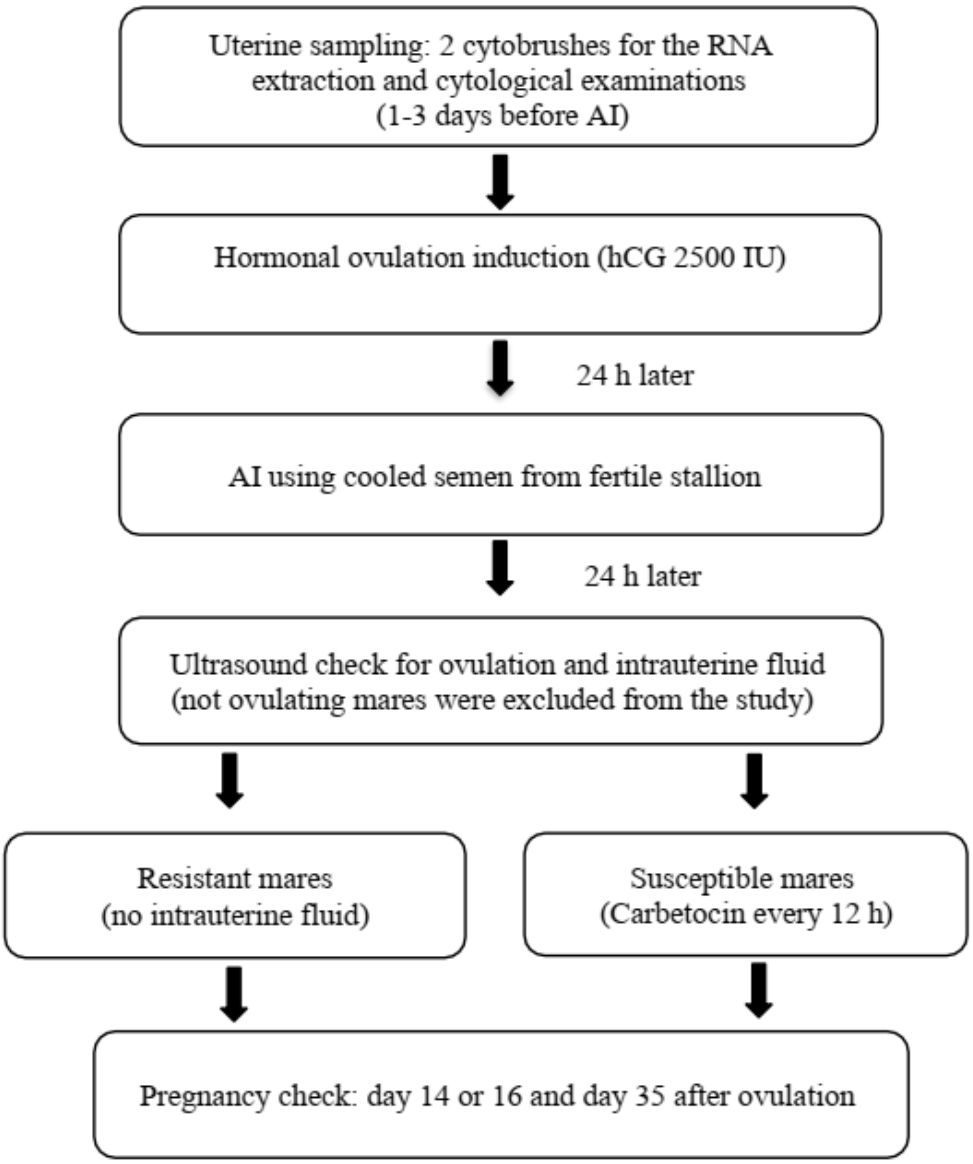


Fig. 1: Time schedule for reproductive management and sampling of the mares. AI=artificial insemination. hCG= human chorionic gonadotropin. IU= international unit.

2.5 Post-breeding evaluation

Twenty-four hours after insemination, the mares were checked by ultrasonography for the onset of ovulation and the amount of intrauterine fluid accumulation. Mares with detectable intrauterine fluids were checked again every 12 h and treated according to the amount and echogenicity of intrauterine fluids either with an intramuscular injection of 140 µg Carbetocin (Depotocin®, Veyx GmbH, Schwarzenborn, Germany) in case of anechogenic intrauterine fluid ≥ 2 cm in diameter or with intra-uterine lavage using 1-3 liters of warm (37^{O} C) sterile physiological saline solution followed directly by Carbetocin injection in case of echogenic intrauterine fluid. Mares were classified as susceptible to PBIE if they showed delayed uterine clearance with ≥ 2 cm of intrauterine fluid 24 hours after the AI. Mares with intrauterine fluid were checked and treated every twelve-hours (i.e. 36, 48, 60, 72 hours post-AI) until no fluid was detectable.

2.6 Pregnancy check

The first pregnancy check was performed on Day14 (in case of double ovulation or Day 16 in case of single ovulation (Day 0 = day of ovulation). A second pregnancy check was performed 35 (±2) days after ovulation.

2.7 Assessment of the samples

2.7.1 Cytological assessment

The cell smears mounted on glass slides were stained with Diff-Quick (RAL DIFF-QUIK™, Siemens Healthineers, UK) according to the manufacturer's instructions. Briefly, the air-dried slides were dipped five times (1 sec. each) into fixative (methanol), solution 1 (xanthene), and solution 2 (thiazine), respectively. Afterwards, the slides were rinsed with distilled water and air-dried before microscopic assessment.

Under a light microscope (Olympus CH2, Olympus, Tokyo, Japan) the slides were evaluated as described by Ferris et al. (2015). The initial evaluation was conducted at low magnification (4X or 10X) to determine the diagnostic value of the slides (enough cellularity to provide accurate interpretation), if the slides were of high diagnostic value they were subsequently evaluated at a magnification of 400X over at least ten high-power fields (HPF) to determine the different cell types, particularly polymorphonuclear neutrophils (PMNs). If the slides were found to be

of insufficient cellularity to be of diagnostic value, new samples were collected on the same day.

The average number of PMNs /HPF was determined and the inflammatory condition of endometrium was categorized as a) normal endometrium, if there were no PMNs, b,) mild inflammation if there were 1 to 2 PMNs, c) moderate inflammation if there were 3 to 5 PMNs, and d) severe inflammation if there were > 5 PMNs per HPF (Ferris et al. 2015).

2.7.2 RNA extraction and quality measurement

Total RNA extraction was performed for 27 samples with the best comparability (nine samples were selected from each SP, SNP, and RP groups), the mares had almost the same age, reproductive status and were inseminated with the same stallions. According to the manufacturer's instructions, total RNA was extracted using the Qiagen RNeasy micro kit (Qiagen, Hilden, Germany) based on the protocol for purifying total RNA from animal and human tissue. Step number three in the manufacture protocol was the start of the procedures in the laboratory as the first two steps were done already after collecting the cytobrushes.
The concentration and purity were measured by spectrophotometry using NanoDrop One (Thermo Fisher Scientific, Waltham, United States), while the quality of each RNA sample was measured using Agilent 2100 Bioanalyzer RNA 6000 Nano assays (Agilent Technologies, Waldbronn, Germany).

2.7.3 RNA-sequencing and data analysis

The RNA isolates were processed by standard Illumina mRNA sequencing using the Illumina Truseq stranded mRNA protocol, which was performed by the Functional Genomic Center Zurich (FGCZ), a core facility of the University of Zurich and ETH Zurich. The samples were barcoded and pooled and the pool was sequenced on an Illumina Novaseq 6000 (approximately, 30 million 100 bp single end reads per sample). The obtained sequence reads (Fastq files) were processed and mapped using a data analysis established on a local Galaxy server installation (ETH Zurich, Animal Physiology group) according to Blankenberg et al. 2010. Briefly, the Fastq files were subjected to quality control checking firstly utilizing 'Fast QC' and 'Multi QC' tools, the adapter sequence was trimmed (for shorter

fragments where sequencing runs into the adapter) and 5 bp were removed from the 5′ end of the reads using Trim Galore (Kim et al. 2015). All the sequence reads were mapped to the reference equine genome assembly (EquCab3.0) from the National Center for Biotechnology Information (NCBI) by using the HISAT2 tool. Feature Counts tool was used to count the number of sequence reads for the annotated genes, while Column Join on data tool was used to create a count table containing all samples. This table was filtered to remove genes with negligible read counts by using the counts per million (CPM) per sample filtering tool (Chen et al. 2017).

The resulting read count table with CPM was used for statistical analysis in EdgeR (using GLM_robust) to identify the differentially expressed genes (DEGs) between susceptible pregnant, susceptible non-pregnant and resistant pregnant (Robinson et al. 2010). All DEGs between mare groups were filtered with a false discovery rate lower than 0.2 (FDR <20 %) using its corresponding p-value as a cut-off to compare the results efficiently.

The online tool MAdb (Gene Symbol match, Ensembl compare database release 95, Blast, https://madb.ethz.ch/) (Bick et al. 2019) was used to acquire the corresponding human gene symbols and gene details for the DEGs. Then, these DEGs were subjected to hierarchical cluster analysis in MultiExperiment Viewer (MeV) using the HCL tool (Saeed et al. 2003). The functional classification and pathway analyses of DEGs were performed in the Metascape online tool (https://metascape.org/) separately for lower and higher expressed genes in mare groups (Zhou et al. 2019).

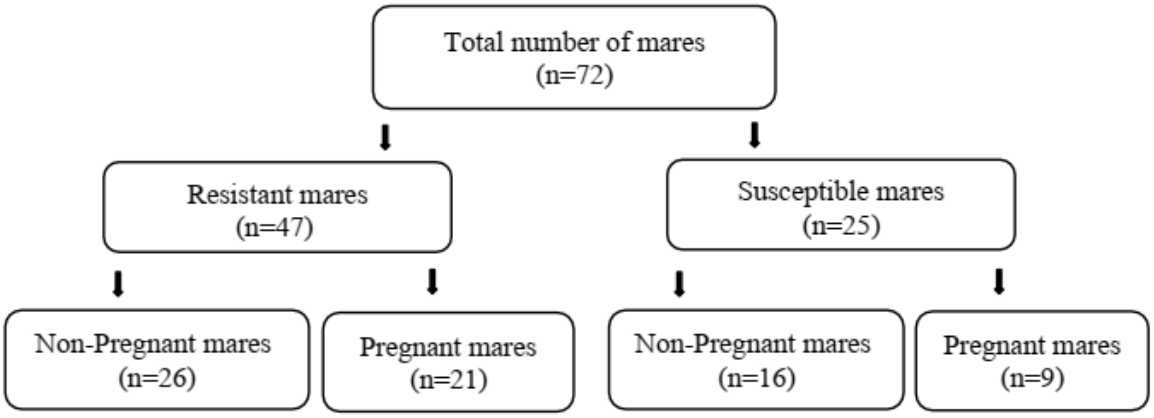


Fig. 2: Grouping of the mares according to susceptibility to persistent breeding induced endometritis and the result of pregnancy diagnosis

2.8 Statistical analysis of the clinical data

The correlation between the following variables: cytology results, intrauterine fluid accumulation and pregnancy results was calculated in SPSS v.26 using Pearson's coefficient test.

3. Results

3.1 Clinical findings

From the 81 mares examined at the beginning of the study, 9 mares did not ovulate within 48 hours after the hormonal induction of ovulation. Therefore, these 9 mares were excluded from the study. Twenty-four susceptible mares showing anechogenic intrauterine fluid were treated using a Carbetocin injection 2-4 times in a 12-hour base. Only one susceptible mare developed echogenic intrauterine fluid that was treated using intrauterine lavage followed afterwards by Carbetocin injections, the injection was repeated until the fluids disappeared.

36% of susceptible mares and 44.7% of resistant mares became pregnant. Three of the resistant pregnant mares had double ovulation, but none of them showed twins in the first pregnancy check. Only one pregnant mare from the resistant group lost the conceptus between the first and second pregnancy checks. All other mares with a positive pregnancy diagnosis on Days 14 or 16 were also pregnant around Day 35.

3.2 Cytological assessment

At the time of sampling, only 32% of susceptible mares showed a mild degree of uterine inflammation, while 38% of the resistant had a mild and 12 % a moderate uterine inflammation. In only 12% of the susceptible mares with positive cytology, a conceptus could be noticed during the pregnancy checks, while 21% of resistant mares with positive cytology became pregnant.

There was a weak positive correlation between positive cytology results and the amount of uterine fluid accumulation ($r = 0.25$; $P < 0.05$). No significant correlation was determined between positive cytology and pregnancy results ($r = -0.02$; $P > 0.05$).

3.3 RNA concentration and quality measurement

The cytobrush technique provided uterine samples that were sufficient to yield an acceptable quantity of good quality RNA suitable for subsequent transcriptomic analysis. The total RNA concentration ranged from 15 to 731 ng/ µl, in addition, the

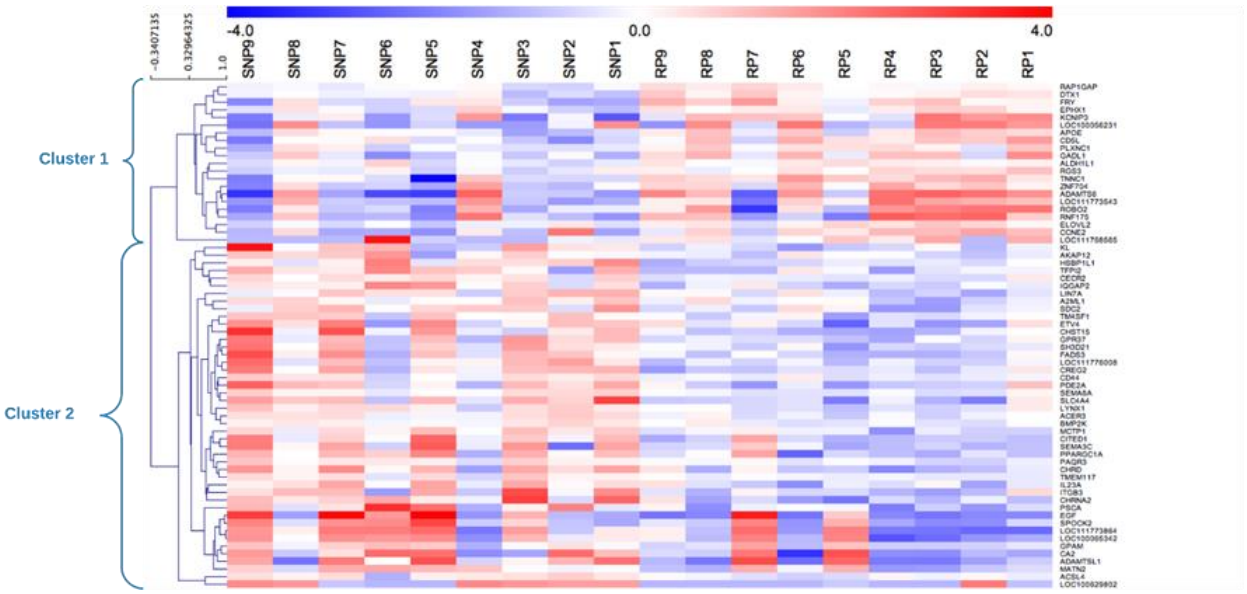
A260/A230 ratio was between 0.03 and 1.85 and the sample RNA Integrity Numbers (RIN) were between 8.6 and 10. After quality filtering of the fastq files, the results of the RNA sequencing revealed library sizes between 20.6 and 78.5 million reads per sample, with an average of 45.1 million reads. After sorting genes with neglectable read counts, a total of 14,725 genes were identified and used for differential gene expression analysis.

3.4 Identification of differentially expressed genes between susceptible mares that did not become pregnant and resistant mares that became pregnant

The uterine transcriptome profiles showed significant variations between susceptible non- pregnant (SNP) and resistant pregnant (RP) mares. Sixty-six differentially expressed genes (DEGs) (FDR<0.2) have been identified among the two groups of mares, 21 genes were expressed significantly lower, while 45 were expressed significantly higher in SNP mares compared to RP mares (Fig. 3).

The expressions of ADAM metallopeptidase with thrombospondin type 1 motif 6 (ADAMTS6), uncharacterized LOC111768565 and ring finger protein 175 (RNF175) mRNAs were lower while the expressions of nucleoside diphosphate kinase B pseudogene (LOC100629802), epidermal growth factor (EGF) and ADAMTS like 1(ADAMTSL1) mRNAs showed higher levels in SNP mares compared to RP mares.
The hierarchical cluster analysis of the DEGs showed a separation of lower expressed (cluster1) and higher expressed (cluster 2) DEGs in SNP mares compared to RP mares (Fig. 3 a), while there is a partial separation between the mare groups (Fig. 3). The lower and higher expressed DEGs in SNP mares compared to RP mares are listed in Tab. 1 and 2.

a)



b)

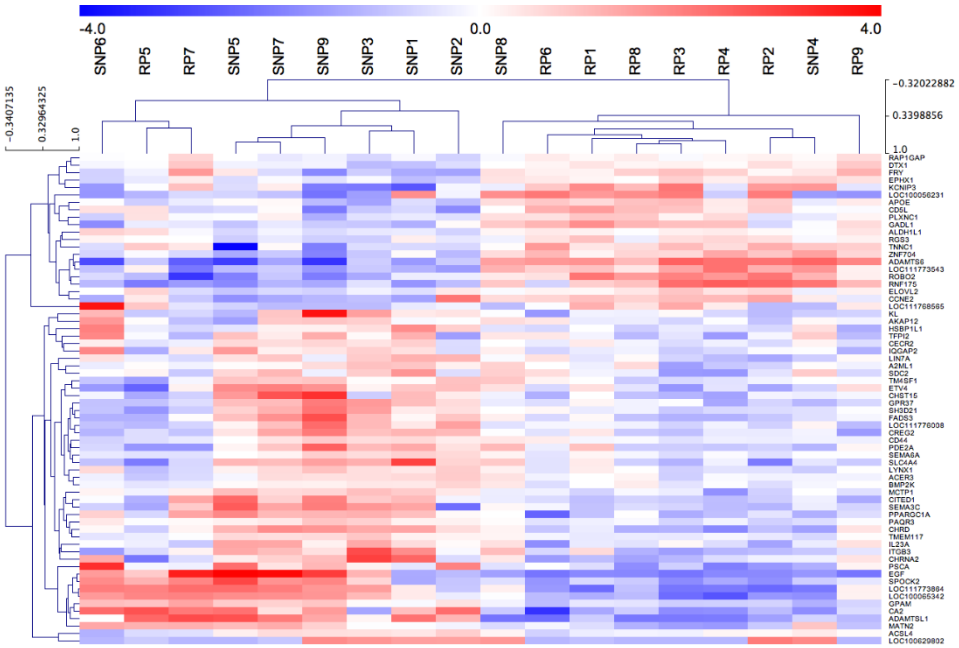


Fig. 3: a) Heat map of hierarchical cluster analysis for DEGs between SNP mares and RP mares (FDR<0.2). Each line represents a gene, each column one sample. Red colors represent higher, while blue colors represent a lower expression of the gene compared to the mean of all samples (mean-centered values in log2 scale), the intensity of the red/blue colors represent the level of gene expression b) Heat map analysis of the mares.

Tab. 1: Genes with lower expression in susceptible mares that did not become pregnant compared to resistant mares that became pregnant

Gene Symbol	Gene ID	Description	Human ID	logFC	*P* -value	FDR
ADAMTS6	100050673	ADAM thrombospondin type 1 motif 6	11174	-2.99	0.0000	0.0092
ALDH1L1	100061108	aldehyde dehydrogenase 1 family member L1	10840	-0.52	0.0006	0.1549
APOE	100065481	apolipoprotein E	348	-0.92	0.0005	0.1458
CCNE2	100055302	cyclin E2	9134	-1.66	0.0004	0.1399
CD5L	100054450	CD5 molecule like	922	-1.28	0.0004	0.1395
DTX1	100051299	deltex E3 ubiquitin ligase 1	1840	-0.73	0.0002	0.1208
ELOVL2	100063624	ELOVL fatty acid elongase 2	54898	-0.74	0.0000	0.0231
EPHX1	100054269	epoxide hydrolase 1	2052	-0.69	0.0004	0.1339
FRY	100062217	FRY microtubule binding protein	10129	-1.31	0.0000	0.0275
GADL1	100057562	glutamate decarboxylase like1	339896	-1.76	0.0007	0.1756
KCNIP3	100062637	potassium voltage-gated channel interacting protein 3	30818	-1.98	0.0003	0.1285
LOC100056231	100056231	saoe class I histocompatibility antigen	LOC100056231	-2.02	0.0005	0.1442
LOC111768565	111768565	uncharacterized LOC111768565	LOC111768565	-2.81	0.0002	0.0952
LOC111773543	111773543	uncharacterized LOC111773543	LOC111773543	-2.17	0.0003	0.1328
PLXNC1	100064706	plexin C1	10154	-0.73	0.0009	0.1980
RAP1GAP	100071716	RAP1 GTPase activating protein	5909	-0.47	0.0003	0.1327
RGS3	100052488	regulator of G protein signaling 3	5998	-0.71	0.0000	0.0306
RNF175	100069989	ring finger protein 175	285533	-2.25	0.0003	0.1285
ROBO2	100068957	roundabout guidance receptor 2	6092	-1.89	0.0001	0.0928
TNNC1	100051811	troponin C1	7134	-1.39	0.0000	0.0326
ZNF704	100057981	zinc finger protein 704	619279	-1.26	0.0000	0.0306

Log FC: logarithm fold change, *P*-value: Probability value, FDR: false discovery rate.

Tab. 2: Genes with higher expression in susceptible mares that did not become pregnant compared to resistant mares that became pregnant

Gene Symbol	Gene ID	Description	Human ID	logFC	*P*- value	FDR
A2ML1	100061421	alpha-2-macroglobulin like 1	144568	0.71	0.0004	0.1435
ACER3	100063572	alkaline ceramidase 3	55331	0.65	0.00089	0.1986
ACSL4	100054459	acyl-CoAsynthetase long chain 4	2182	0.61	0.0001	0.0928
ADAMTSL1	100063467	ADAMTS like 1	92949	2.62	0.0000	0.0275
AKAP12	100063263	A-kinase anchoring protein 12	9590	0.81	0.0009	0.1980
BMP2K	100059466	BMP2 inducible kinase	55589	0.47	0.0006	0.1620
CA2	100050059	carbonic anhydrase 2	760	2.37	0.0005	0.1442
CD44	100034221	CD44 molecule	960	0.59	0.0001	0.0928
CECR2	100055261	CECR2 histone acetyl-lysine reader	27443	0.75	0.0000	0.0275
CHRD	100067164	Chordin	8646	1.91	0.0000	0.0164
CHRNA2	100060380	Cholinergic-receptor nicotinic alpha 2 subunit	1135	2.35	0.0000	0.0275
CHST15	100057594	carbohydrate sulfotransferase 15	51363	1.66	0.0002	0.0938
CITED1	100051456	Cbp/p300 interacting transactivator Glu/Asp rich carboxy-terminal domain 1	4435	2.54	0.0000	0.0231
CREG2	100059003	cellular repressor of E1A stimulated genes 2	200407	1.82	0.0001	0.0928
EGF	106782874	epidermal growth factor	1950	5.22	0.0000	0.0202
ETV4	100065121	ETS variant transcription factor 4	2118	1.42	0.0007	0.1756
FADS3	100059870	fatty acid desaturase 3	3995	2.48	0.0005	0.1442
GPAM	100068677	glycerol-3-phosphate acyltransferase, mitochondrial	57678	0.93	0.0004	0.1396
GPR37	100071585	G-protein receptor 37	2861	1.40	0.0006	0.1549
HSBP1L1	102150197	heat shock factor binding protein 1 like 1	440498	1.37	0.0006	0.1620
IL23A	100034230	interleukin 23 subunit alpha	51561	1.60	0.0004	0.1399
IQGAP2	100073279	IQcontaining GTPase activating protein 2	10788	1.03	0.0000	0.0306
ITGB3	100009709	integrin subunit beta 3	3690	1.65	0.0005	0.1442
KL	100064836	Klotho	9365	1.57	0.0008	0.1893
LIN7A	100050683	lin-7 homolog A, crumbs cell complex	8825	0.96	0.0003	0.1327
LOC100065342	100065342	UDP-glucuronosyltransferase 1-1	54658	1.84	0.0004	0.1395
LOC100629802	100629802	nucleoside diphosphate kinase B pseudogene	LOC100629802	5.28	0.0000	0.0017
LOC111773864	111773864	UDP-glucuronosyltransferase 1-10-like	LOC111773864	2.38	0.0005	0.1442
LOC111776008	111776008	uncharacterized LOC111776008	LOC111776008	1.33	0.0003	0.1328

Log FC: logarithm fold change, *P*- value: Probability value, FDR: false discovery rate.

Tab. 2: Genes with higher expression in susceptible mares that did not become pregnant compared to resistant mares that became pregnant

Gene Symbol	Gene ID	Description	Human ID	logFC	P- value	FDR
MATN2	100055555	matrilin 2	4147	1.46	0.0007	0.1756
MCTP1	100073232	multiple C2 and transmembrane domain containing 1	79772	1.03	0.0003	0.1285
PAQR3	100059505	progestin adipo Q receptor 3	152559	0.59	0.0003	0.1290
LYNX1	100066475	Ly6/neurotoxin 1	66004	0.87	0.0003	0.1285
PDE2A	100052619	phosphodiesterase 2A	5138	1.45	0.0003	0.1328
PPARGC1A	100055716	PPARG coactivator 1 alpha	10891	1.52	0.0000	0.0190
PSCA	100063721	prostate stem cell antigen	8000	1.77	0.0005	0.1442
SDC2	100059727	syndecan 2	6383	1.15	0.0002	0.0997
SEMA3C	100054706	semaphorin 3C	10512	1.78	0.0000	0.0275
SEMA6A	100073147	semaphorin 6A	57556	0.62	0.0000	0.0384
SH3D21	100069289	SH3 domain containing 21	79729	1.19	0.0005	0.1442
SLC4A4	100049977	solute carrier family 4 member 4	8671	1.83	0.0000	0.0032
SPOCK2	100063582	SPARC (osteonectin), kazal like domains 2	9806	1.76	0.0006	0.1575
TFPI2	100051361	tissue factor pathway inhibitor 2	7980	1.47	0.0001	0.0928
TM4SF1	100050742	transmembrane 4 L six family member 1	4071	0.92	0.0003	0.1328
TMEM117	100050185	transmembrane protein 117	84216	0.56	0.0005	0.1458

3.5 Overrepresented functional categories

The corresponding human NCBI Entrez gene IDs of the lower and higher expressed DEGs were uploaded separately to the online Metascape Enrichment Analysis Tool to identify their over-represented functional categories (Fig.4).

For genes with lower expression in the SNP group compared to the RP group, functional categories such as 1) developmental processes "Artery and cardiac chamber development", 2) positive regulation of biological process "axon guidance, positive regulation of cell development, and regulation of cell differentiation " and 3) locomotion “Hallmark of xenobiotic metabolism and enzyme activator activity " were overrepresented, while the higher expressed DEGs were enriched for 1) metabolic processes "glycosaminoglycan and mucopolysaccharide metabolic process ", 2) developmental process "tissue remodeling, receptor complex and regulation of peptidyl-tyrosine phosphorylation" 3) response to stimulus "tissue morphogenesis" and 4) signaling "cellular response to growth factor stimulus and negative regulation of cell differentiation" functional categories in SNP compared to RP mares (Tab. 3).

Tab. 3: Metascape enrichment analysis of DEGs in susceptible mares that did not become pregnant compared to resistant mares that became pregnant

Most informative categories of Metascape enrichment analysis	LogP	Genes
Lower expressed DEGs		
Artery development	-4.26	APOE, ROBO2, ADAMTS6, TNNC1
Axon guidance, positive regulation of cell development, regulation of cell morphogenesis involved in differentiation.	-3.56	RGS3, ROBO2, PLXNC1, APOE, RAP1GAP, DTX1
. Hallmark of xenobiotic metabolism, enzyme activator activity.	-3.39	APOE, EPHX1, RAP1GAP, RGS3, CCNE2
Small molecule catabolic process	-2.39	APOE, ALDH1L1, GADL1
Blood circulation, circulatory system process	-2.15	APOE, TNNC1, KCNIP3
Higher expressed DEGs		
Glycosaminoglycan metabolic process, mucopolysaccharide metabolic process	-5.11	CD44, EGF, SDC2, SPOCK2, CHST15
Microvillus, tissue remodeling, receptor complex regulation of peptidyl-tyrosine phosphorylation	-4.79	CA2, CD44, ITGB3, IQGAP2, SEMA3C, IL23A, EGF, MATN2, TFPI2, SDC2,
Tissue morphogenesis, morphogenesis of a branching epithelium.	-4.74	CA2, CD44, EGF, ITGB3, MATN2, CITED1, CHRD, SEMA3C, GPAM
Cellular response to growth factor stimulus, negative regulation of cell differentiation.	-4.62	CD44, ITGB3, CITED1, PDE2A, CHRD, KL, PPARGC1A, SEMA6A, BMP2K, SEMA3C, PAQR3, EGF, ACSL4, TM4SF1
NABA MATRISOME ASSOCIATED, regulation of chemotaxis.	-4.42	EGF, SDC2, CHRD, SEMA3C, IL23A, SEMA6A, ADAMTSL1, A2ML1, ITGB3, AKAP12, SPOCK2, KL, LYNX1,
Regulation of MAPK cascade, regulation of ERK1 and ERK2 cascade.	-4.42	CD44, EGF, GPR37, PSCA, KL, AKAP12, SEMA6A, PAQR3
MAPK family signaling cascades, MAPK1/MAPK3 signaling, regulation of endocytosis.	-3.84	EGF, ETV4, ITGB3, KL, PAQR3, BMP2K, MCTP1, IQGAP2, SDC2, SEMA6A
Regulation of signaling receptor activity, negative regulation of MAPK cascade,	-3.64	EGF, PSCA, PPARGC1A, LYNX1, CD44, PDE2A, SEMA6A, PAQR3
Pigment metabolic process, cellular response to lipopolysaccharide, steroid, and toxic substance.	-3.54	CITED1, PPARGC1A, UGT1A1, GPAM, AKAP12, PDE2A, GPR37, CA2, SDC2, KL, IL23A

Tab. 3: Metascape enrichment analysis of DEGs in susceptible mares that did not become pregnant compared to resistant mares that became pregnant

Higher expressed DEGs	LogP	Genes
Positive regulation of carbohydrate metabolic process, positive regulation of small molecule metabolism.	-3.38	EGF, SLC4A4, PPARGC1A, KL, CHST15, UGT1A1, ACSL4, FADS3, GPAM, SDC2, PDE2A, CITED1, SPOCK2, ACER3, CECR2
Organic hydroxy compound biosynthetic process.	-2.89	GPR37, CITED1, ACER3, GPAM
Chemical synaptic transmission, trans-synaptic signaling.	-2.85	CA2, CHRNA2, LIN7A, AKAP12, LYNX1, MCTP1, ETV4
Synaptic membrane.	-2.35	CHRNA2, ITGB3, PDE2A, LIN7A
Calcium ion binding.	-2.15	EGF, MATN2, SPOCK2, ACER3, MCTP1
Diseases of metabolism.	-2.06	SDC2, UGT1A1, ADAMTSL1
Dendrite development.	-2.04	ACSL4, MATN2, SDC2

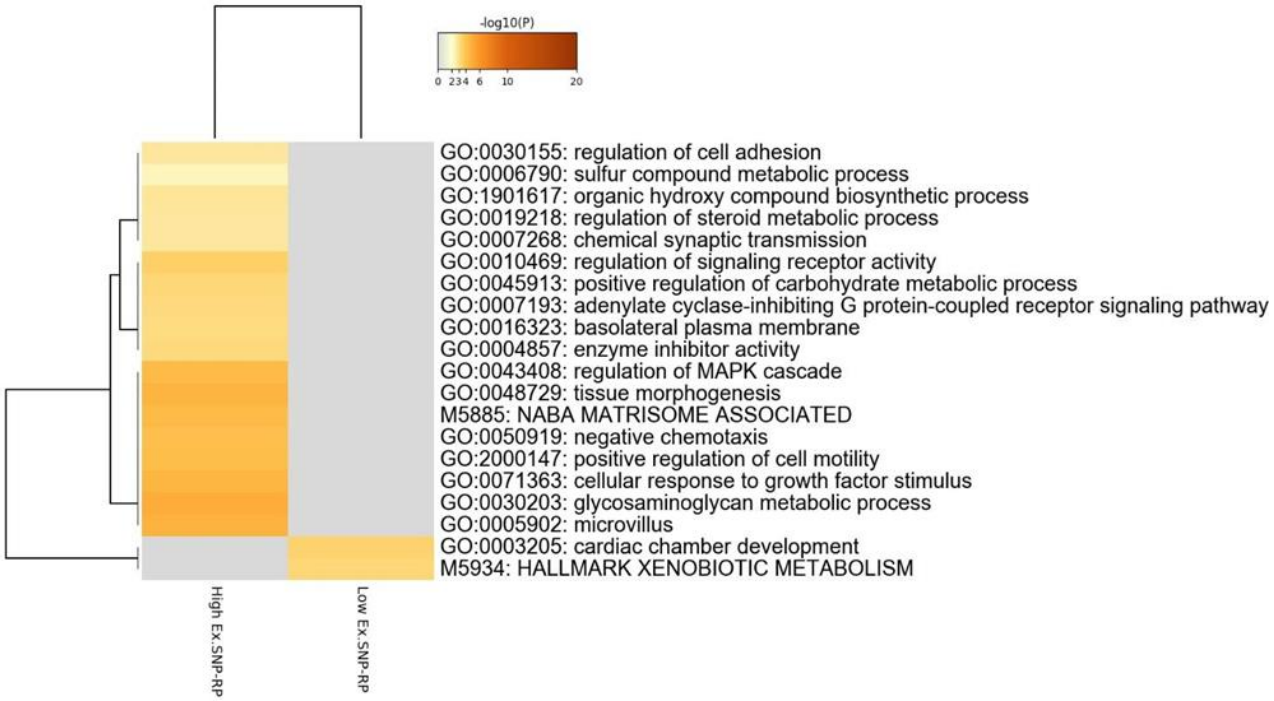


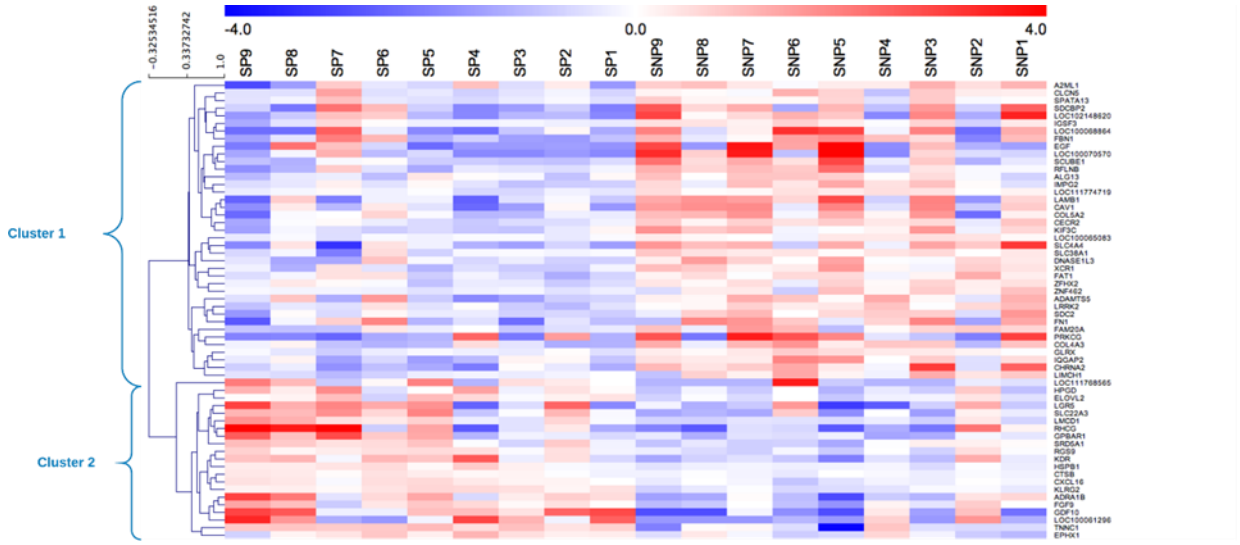
Fig. 4: Metascape analysis: most enriched categories of genes with lower and higher expression in susceptible mares that did not become pregnant compared to resistant mares that became pregnant. The color intensity represents the level of functional enrichment.

3.6 Identification of differentially expressed genes between susceptible mares that become pregnant and susceptible mares that did not become pregnant

A total of sixty DEGs (FDR<0.2) were detected in the comparison of SP mares and SNP mares, thirty- nine were significantly lower, whereas twenty-one were higher expression in SP mares relative to SNP mares. The expression of aldo-keto reductase family 1 member C23 (LOC100070570), protein kinase C gamma (PRKCG) and epidermal growth factor (EGF) mRNAs was lower, while the expression of membrane-spanning 4-domains subfamily A member 4A (LOC100061296), growth differentiation factor 10 (GDF10) and Rh family C glycoprotein (RHCG) mRNAs was higher in SP mares compared to SNP mares.

The hierarchical cluster analysis demonstrates separation of the lower-expressed (cluster 1) and higher-expressed (cluster 2) DEGs in SP mares vs. SNP mares (Fig. 5a), however, there is only a partial separation between the mare groups (Fig. 5b). The DEGs of lower and higher expression are listed in Tab. 4 and 5.

a)



b)

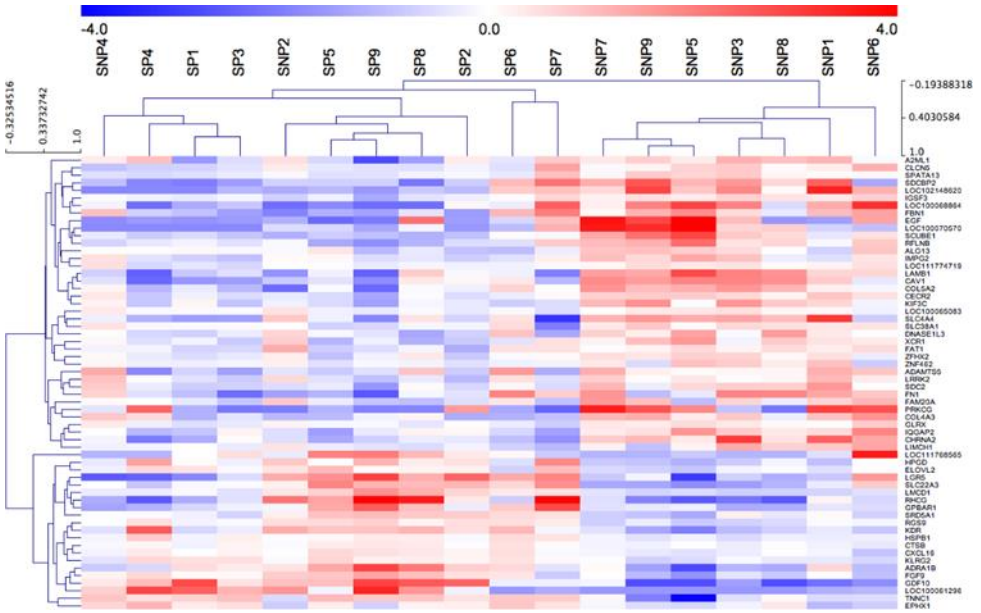


Fig. 5: a) Heat map of hierarchical cluster analysis for DEGs between SP mares and SNP mares (FDR<0.2). Each line represents a gene, each column one sample. Red colors represent higher while, blue colors represent the lower expression of the gene compared to the mean of all samples (mean-centered values in log2 scale), the intensity of the red/blue colors represent the level of gene expression b) Heat map analysis of mares.

Tab. 4: Genes with lower expression in susceptible mares that become pregnant compared to susceptible mares that did not become pregnant.

Gene Symbol	Gene ID	Description	Human ID	logFC	*P*- value	FDR
A2ML1	100061421	alpha-2-macroglobulin like 1	144568	-1.17	0.0003	0.0963
ADAMTS5	100066005	ADAM throm motif 5	11096	-1.60	0.0002	0.0834
ALG13	100058763	acetylglucosaminyltransferase subunit 13	79868	-0.83	0.0006	0.1630
CAV1	100055975	caveolin 1	857	-1.99	0.0000	0.0117
CECR2	100055261	CECR2 histone acetyl-lysine reader	27443	-1.02	0.0000	0.0088
CHRNA2	100060380	cholinergic receptor nicotinic alpha 2 subunit	1135	-2.47	0.0000	0.0117
CLCN5	100052289	chloride voltage-gated channel 5	1184	-0.76	0.0002	0.0812
COL4A3	100037402	collagen type IV alpha 3 chain	1285	-1.51	0.0001	0.0579
COL5A2	100054421	collagen type V alpha 2 chain	1290	-1.28	0.0004	0.1193
DNASE1L3	100057863	deoxyribonuclease 1 like 3	1776	-1.97	0.0002	0.0812
EGF	106782874	epidermal growth factor	1950	-3.50	0.0004	0.1265
FAM20A	100062707	FAM20A golgi associated secretory pseudokinase	54757	-1.20	0.0001	0.0633
FAT1	100050525	FAT atypical cadherin 1	2195	-0.76	0.0001	0.0756
FBN1	100055741	fibrillin 1	2200	-1.62	0.0000	0.0484
FN1	100034189	fibronectin 1	2335	-1.62	0.0008	0.1952
GLRX	100064937	Glutaredoxin	2745	-0.65	0.0001	0.0756
IGSF3	100059648	immunoglobulin superfamily member 3	3321	-0.60	0.0009	0.2132
IMPG2	100061979	interphotoreceptor matrix proteoglycan 2	50939	-0.98	0.0002	0.0834
IQGAP2	100073279	IQ motif containing GTPase activating protein 2	10788	-1.08	0.0001	0.0756
KIF3C	100055794	kinesin family member 3C	3797	-1.27	0.0000	0.0210
LAMB1	100060360	laminin subunit beta 1	3912	-1.96	0.0000	0.0279
LIMCH1	100063373	LIM and calponin homology domains 1	22998	-0.93	0.0000	0.0473
LOC100065083	100065083	solute carrier family 35 member E2	728661	-0.47	0.0001	0.0579
LRRK2	100052935	leucine rich repeat kinase 2	120892	-1.29	0.0004	0.1193
PRKCG	100062549	protein kinase C gamma	5582	-3.95	0.0000	0.0473
RFLNB	100072318	refilin B	359845	-1.31	0.0001	0.0642

Tab. 4: Genes with lower expression in susceptible mares that become pregnant compared to susceptible mares that did not become pregnant.

Gene Symbol	Gene ID	Description	Human ID	logFC	*P*-value	FDR
LOC100068864	100068864	major allergen Can f 1-like	3933	-3.21	0.0004	0.1214
LOC100070570	100070570	aldo-keto reductase family 1 member C23	LOC100070570	-4.09	0.0001	0.0586
LOC102148620	102148620	Antileukoproteinase	128488	-3.25	0.0002	0.0834
LOC111774719	111774719	Uncharacterized LOC111774719	LOC111774719	-0.60	0.0000	0.0117
SCUBE1	100071128	signal peptide, CUB domain and EGF like domain containing 1	80274	-2.77	0.0001	0.0756
SDC2	100059727	syndecan 2	6383	-1.12	0.0001	0.0566
SDCBP2	100053209	syndecan binding protein 2	27111	-2.44	0.0003	0.1017
SLC38A1	100050256	solute carrier family 38-member 1	81539	-0.69	0.0002	0.0776
SLC4A4	100049977	solute carrier family 4-member 4	8671	-2.10	0.0000	0.0117
SPATA13	100058228	spermatogenesis associated 13	221178	-0.59	0.0008	0.2011
XCR1	100065485	X-C motif chemokine receptor 1	2829	-1.52	0.0002	0.0776
ZFHX2	100052278	zinc finger homeobox 2	85446	-0.69	0.0004	0.1256

Log FC: logarithm fold change, *P*- value: Probability value, FDR: false discovery rate.

Tab. 5: Genes with higher expression in susceptible mares that become pregnant compared to susceptible mares that did not become pregnant.

Gene Symbol	Gene ID	Description	Human ID	logFC	*P*-value	FDR
ADRA1B	100071055	adrenoceptor alpha 1B	147	1.83	0.0007	0.1880
CTSB	100060963	cathepsin B	1508	0.38	0.0006	0.1600
CXCL16	100061442	C-X-C motif chemokine ligand 16	58191	0.55	0.0001	0.0510
ELOVL2	100063624	ELOVL fatty acid elongase 2	54898	0.66	0.0007	0.1880
EPHX1	100054269	epoxide hydrolase 1	2052	0.83	0.0001	0.0739
FGF9	100050353	fibroblast growth factor 9	2254	0.94	0.0004	0.1256
GDF10	100063615	growth differentiation factor 10	2662	3.78	0.0000	0.0117
GPBAR1	100055590	G protein-coupled bile acid receptor 1	151306	1.88	0.0006	0.1717
HPGD	100009687	15-hydroxyprostaglandin dehydrogenase	3248	1.04	0.0003	0.1028
HSPB1	100059763	heat shock protein family B (small) member 1	3315	0.55	0.0001	0.0642
KDR	100033959	kinase insert domain receptor	3791	1.48	0.0000	0.0117
KLRG2	102148132	killer cell lectin like receptor G2	346689	0.79	0.0002	0.0775
LGR5	100064565	leucine rich repeat containing G protein-coupled receptor 5	8549	2.10	0.0008	0.2065
LMCD1	100058957	LIM and cysteine rich domains 1	29995	1.00	0.0001	0.0707
LOC100061296	100061296	membrane-spanning 4-domains subfamily A member 4A	51338	4.86	0.0000	0.0117
LOC111768565	111768565	uncharacterized LOC111768565	LOC111768565	3.18	0.0002	0.0776
RGS9	100053338	regulator of G protein signaling 9	8787	0.70	0.0002	0.0776
RHCG	100069692	Rh family C glycoprotein	51458	3.66	0.0003	0.1191
SLC22A3	100058287	solute carrier family 22 member 3	6581	1.62	0.0000	0.0282
SRD5A1	100071450	steroid 5 alpha-reductase 1	6715	0.86	0.0007	0.1938
TNNC1	100051811	troponin C1, slow skeletal and cardiac type	7134	1.28	0.0000	0.0473

Log FC: logarithm fold change, *P*-value: Probability value, FDR: false discovery rate.

3.7 Overrepresented functional categories

The overrepresented functional categories of genes with lower expression in SP mares compared to SNP mares were 1) cellular component organization "degradation of the extracellular matrix (ECM), focal adhesion, ECM-receptor interaction, PI3K-Akt signaling pathway", 2) regulation of biological processes "negative regulation of proteolysis and hydrolase activity" and 3) developmental processes "Cell junction assembly and positive regulation of peptidase activity". While the genes with higher expression were enriched for 1) response to stimulus "Cellular response to growth factor stimulus, epithelial cell proliferation, positive regulation of MAPK cascade and regulation of vasculature development ", 2) signaling "response to toxic substance, response to estradiol, urogenital system development" 3) cell proliferation "second- messenger-mediated signaling" and 4) reproductive processes "Developmental process involved in reproduction, collagen-containing extracellular matrix " functional categories in SP compared to SNP mares (Tab. 6, Fig. 6).

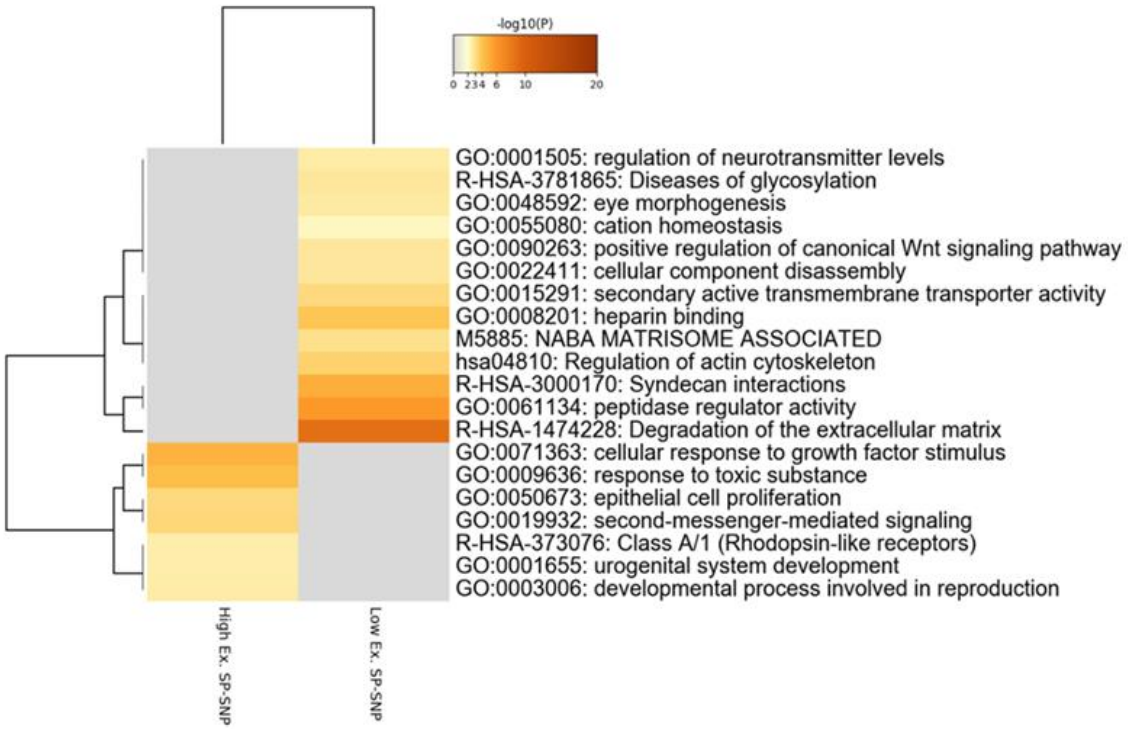


Fig. 6: Metascape analysis: most enriched categories of genes with lower and higher expression in susceptible mares that become pregnant compared to susceptible mares that did not become pregnant. The color intensity represents the level of functional enrichment.

Tab.6: Metascape enrichment analysis of DEGs susceptible mares that become pregnant compared to susceptible mares that did not become pregnant.

Most informative categories of Metascape enrichment analysis	LogP	Genes
Lower expressed DEGs		
Degradation of the extracellular matrix, ECM organization, focal adhesion, ECM-receptor interaction, PI3K-Akt signaling pathway.	-8.80	COL4A3, COL5A2, FBN1, FN1, LAMB1, ADAMTS5, SCUBE1, SDC2, CAV1, IMPG2, EGF, PRKCG, FAM20A, LRRK2, FAT1
Peptidase regulator activity, negative regulation of proteolysis, negative regulation of hydrolase activity.	-5.97	CAV1, COL4A3, FN1, LCN1, WFDC12, A2ML1, PRKCG, LRRK2, IQGAP2
Syndecan interactions, positive regulation of peptidase activity, cell junction assembly.	-5.03	COL5A2, FN1, SDC2, CAV1, PRKCG, EGF, KIF3C, COL4A3, GLRX, LIMCH1
Rho GTPase binding, positive regulation of proteolysis, cation homeostasis.	-4.18	CAV1, IQGAP2, LRRK2, SPATA13, COL4A3, EGF, FN1, PRKCG, CHRNA2, GLRX, SLC4A4, FAM20A, ZNF462, XCR1
Regulation of actin cytoskeleton, platelet activation, signaling and aggregation, response to elevated platelet cytosolic $Ca2^{+}$	-3.53	EGF, FN1, IQGAP2, SPATA13, PRKCG, LRRK2
Actin-based cell projection, actin filament organization.	-3.49	FAT1, IQGAP2, LRRK2, SPATA13, LIMCH1, RFLNB, COL5A2, CAV1
Secondary active transmembrane transporter activity, inorganic cation transmembrane transport, anion transmembrane transport.	-3.33	CLCN5, SLC4A4, SLC38A1, SLC35E2B, CAV1, GLRX, XCR1, LCN1
NABA MATRISOME ASSOCIATED, NABA ECM regulators.	-3.05	EGF, SDC2, ADAMTS5, FAM20A, SCUBE1, A2ML1, LAMB1, ALG13
Cellular component disassembly.	-2.81	CAV1, DNASE1L3, ADAMTS5, CECR2, SCUBE1
Eye morphogenesis, calcium ion binding.	-2.80	COL5A2, FAT1, FBN1, DNASE1L3, EGF, SCUBE1
Regulation of neurotransmitter levels, transmission across chemical synapses.	-2.69	CAV1, PRKCG, SLC38A1, LRRK2, CHRNA2
Negative regulation of cell differentiation.	-2.25	CAV1, COL5A2, FBN1, LRRK2, RFLNB, COL4A3

Tab.6: Metascape enrichment analysis of DEGs susceptible mares that become pregnant compared to susceptible mares that did not become pregnant.

Higher expressed DEGs	LogP	Genes
Cellular response to growth factor stimulus, epithelial cell proliferation, positive regulation of MAPK cascade, blood vessel development, regulation of vasculature development.	-4.79	FGF9, GDF10, HPGD, HSPB1, KDR, SRD5A1, LGR5, GPBAR1, ADRA1B, CXCL16
Response to toxic substance, response to estradiol, urogenital system development.	-4.23	EPHX1, HPGD, KDR, SRD5A1, RGS9, LGR5
Second-messenger-mediated signaling, regulation of muscle system process.	-3.37	ADRA1B, KDR, LGR5, LMCD1, TNNC1
Developmental processes involved in reproduction, collagen-containing extracellular matrix, reproductive structure development.	-2.68	CTSB, FGF9, SRD5A1, LGR5, GDF10, HPGD, CXCL16
Class A/1 (Rhodopsin-like receptors)	-2.60	ADRA1B, CXCL16, GPBAR1

4. Discussion

The present study is to the best of our knowledge the first clinical prospective investigation of using RNA-Seq analysis of uterine cytobrush samples to identify RNA markers for the susceptibility to PBIE by comparing the uterine transcriptome profiles in susceptible mares that did not become pregnant (SNP) in comparison to resistant mares that became pregnant (RP). Furthermore, the uterine transcriptome profiles of susceptible mares that became pregnant and susceptible that did not become pregnant (SP) were compared.

4.1 Clinical findings

We included only mares ≥ 10 years, because such mares may have a higher risk of susceptibility to the PBIE (Carnevale and Ginther 1992, Grüninger et al. 1998, Woodward et al. 2012). Older mares are known to have more often a poor vulvar conformation, a cervical dilation and a lower lymphatic drainage, which can be involved in accumulation of intrauterine fluids post AI (Bucca et al. 2008, Canisso et al 2016). Although intrauterine fluid is often associated with a positive cytological finding (Burleson et al. 2010), we found a weak positive association between positive uterine cytology results before AI and the intrauterine fluid accumulations

after AI. Intrauterine fluid is a common clinical sign of endometritis (Allen and Pycock 2010), however only mares with severe uterine inflammation according to cytological findings (>5 PMNs/field) showed an accumulation of intrauterine fluids (Burleson et al. 2010). Moreover, Escherichia coli, one of the most common uterine isolates in the equine uterus (Albihn et al. 2003), is usually not associated with PMNs (Overbeck et al 2011). These observations may explain the low association between positive cytological findings and uterine fluid accumulations in our study.

Besides, we found no positive cytology had no negative effect on pregnancy rates. In contrast, Riddle et al. (2007) reported that presence of >2 PMNs per microscopic high-power field was associated with reduced pregnancy rates. Also, this discrepancy may be due to using different interpretation methods of the cytology slides. Riddle et al. (2007) considered the presence of 0–2 neutrophils per microscopic high-power field as a non-inflamed uterus, while we interpreted findings with 1 to 2 PMNs already as positive.

4.2 Lower expressed genes in susceptible mares that did not become pregnant in comparison to resistant mares that became pregnant

The comparative transcriptome analysis demonstrated differences in mRNA expression profiles among mare groups, 66 DEGs were identified by comparing SNP mares and RP mares and 60 DEGs between SP mares and SNP mares. In the following, selected DEGs potentially important for fertility prediction are discussed. Genes of lower expression in SNP mares were most significantly enriched for biological processes related to the regulation of cell development and differentiation. We suggest that the genes apolipoprotein E (APOE) and Roundabout 2 (ROBO2) may play a role in mares' resistance to PBIE based on their known roles in the regulation of steroid hormone metabolism and neutrophil chemotaxis. Such genes were found to be lower expressed in SNP mares compared to RP mares.

The protein encoded by the gene APOE belongs to the apolipoprotein family which is known as lipid transporters, enzyme co-factors, and receptor ligands (Mahley and Rall 2000, Ganfornina et al. 2008). APOE interacts with the low-density lipoprotein receptor (LDLR). Such interaction is essential for normal catabolism and cellular uptake of triglyceride-rich lipoprotein in peripheral tissues participating in immune modulation, cell migration, neurodevelopment, and synaptic plasticity (Stolt and Bock 2006). Several of these functions are important for reproductive functions especially in the uterus (Kao et al. 2002, Germeyer et al. 2013).

In agreement with our findings, lower levels of fertility have been reported in APOE knock-out mice (Collazo et al. 2012). As APOE regulates steroid hormone metabolism any alteration in the mRNA expression of the APOE gene adversely affects some uterine functions like impairing the implantation process (Germeyer et al. 2013).

In cattle, the expression of APOE is found to be upregulated in the normal endometrium during the estrous phase (Bauersachs et al. 2005). Additionally, this gene is reported to be highly expressed in the normal endometrium of women during the implantation window (Kao et al. 2002). As there is a correlation between APOE mRNA expression and impaired lipid levels (cholesterol, triglyceride, low-density lipoprotein) which may be harmful to mother and fetus (Descamps et al. 2005, Hieronimus et al. 2005, Chuang et al. 2009), it has been proposed that the APOE gene could be a potential factor in the reproductive performance of a woman affecting her opportunity to have a successful pregnancy (McGladdery and Frohlich, 2001).
In addition, it has been demonstrated that the uterus of women of high fecundity (potential fertility) expresses high APOE levels that subsequently improves the half-life levels of reproductive steroid hormones (Jasienska et al. 2015). The expression of the APOE gene in the human endometrium is found to be reduced in the case of endometriosis compared to a healthy endometrium during both the proliferative and secretory phases of the menstrual cycle (Sundqvist et al. 2012).

APOE protein mediates binding and neutralizing the microbial cell wall lipopolysaccharide (LPS), and reportedly suppresses the systemic inflammatory responses to the LPS intravenous injection in wild mice (Wang et al. 2009). In addition, APOE has been proven to decrease the recruitment of neutrophils and macrophages into the bronchoalveolar lavage in wild mice (Ni et al. 2013), indicating potential anti-inflammatory and antioxidant activities of APOE to minimize the severity of inflammatory diseases (Yao et al. 2016).
The protein encoded by the gene ROBO 2 belongs to a superfamily of immunoglobulins which works as transmembrane receptors for SLITs ligands. In several body systems, SLITs mediate cell migration and polarization of different cell types (Tessier-Lavigne and Goodman 1996). Three (SLIT 1–3) proteins bind to each of the four (ROBO 1–4) receptors (Legg et al. 2008). The ROBO2 encoded protein is a SLIT2 transmembrane receptor, which plays a role in axon guidance during nervous system development (Hinck, 2004), as well as in regulating the angiogenesis (Jones et al. 2008).

In women, it has been reported that the SLIT / ROBO pathway may have key roles in the reproductive system (Dickinson et al. 2008, Tong et al. 2019). In agreement with our results, the mRNA expression of the ROBO 2 gene is found to be higher in the uterus in women during their follicular phase of menstrual cycle, indicating the significant role of SLIT / ROBO pathway during normal endometrial development (Duncan et al. 2010).

SLIT / ROBO pathway acts as a powerful inhibitor of chemotaxis of circulating neutrophils towards various inflammatory stimuli, preventing the migration of many forms of inflammatory cells, like neutrophils, macrophages, T-lymphocytes and dendritic cells to various chemo-attractive signals, which could function as a highly effective anti-inflammatory agent (Guan et al. 2007, Prasad et al. 2008, Wu et al. 2001).

Nevertheless, many studies have reported the role of Slit2 / Robo-1 in the control of inflammation, we assume that the binding of SLIT2 to ROBO 2 may exert a similar anti-inflammatory effect, since ROBO 1 and ROBO 2 are structurally identical, besides, SLIT2 has the same affinity for both ROBO1 and ROBO 2 receptors (Dickinson and Duncan 2010).

4.3 Higher expressed genes in susceptible mares that did not become pregnant in comparison to resistant mares that became pregnant

In the SNP mares some genes that enriched for cell-cell and cell-extracellular matrix interactions, glycosaminoglycan metabolism and tissue remodeling were highly expressed. We assume that the higher expression of Cluster domain 44 (CD44), Integrin beta 3 (ITGB3), Epidermal growth factor (EGF) and interleukin-23 subunit alpha (IL23A) genes in SNP compared to RP mares mediates the development and/or maintenance of uterine inflammation.

The gene CD44 is encoded for a cell-surface glycoprotein receptor of many well-known ligands including hyaluronic acid (HA), osteopontin (OPN), collagens, and matrix metalloproteinases (MMPs) (Goodison et al. 1999) that engage in many cellular activities including the activation, recirculation and homing of T-lymphocytes, hematopoiesis, response to bacterial infection, HA uptake/degradation, lymph node homing, prothymocyte homing, cell aggregation, chemokine release and inflammation (Haynes et al. 1991, Lesley et al. 1993), but these CD44 functions depend on their ligand specificity (Weber et al. 1996).

Hyaluronan is a glycosaminoglycan of the extracellular matrix that mainly exerts its function

through binding specifically to CD44. It is a widely distributed adhesion protein that mediates cell-cell and cell-microenvironment interactions (Johnson and Ruffell 2009). HA-CD44 is crucial for the development of inflammatory responses like apoptosis and lymphocyte migration. Additionally, several lines of evidence link CD44 to fibroblast adhesion, migration, and fibrosis development (Evanko et al 2007, Bajorath 2000).
CD44 expression has been immunolocalized in luminal epithelia of equine ovaries, fallopian tubes and uterus, as well as in mesenchymal elements requiring frequent remodeling (e.g., fibroblast blood vessels, myocytes, and tissue walls), in the uterus, CD44 receptor is mainly located on the endometrial surface of the epithelium and stromal leukocytes (Rodriguez et al 2011).

In bovine and porcine species, the expression of CD44 is observed at various stages of oocyte and embryo development and is also implicated in sperm preservation and fertilization (Yokoo et al. 2002, Furnus et al. 2003). Besides, different CD44 isoforms have been found in human reproductive tissues, like cervical epithelium (Woerner et al. 1995) endometrial stromal and epithelial cells (Behzad et al. 1994) follicular fluid (Ohta et al. 2001) placenta, oocytes, early embryos, and sperm cells (Aplin 1997, Goshen et al. 1996, Ohta et al. 1999, Bains et al. 2002).

It is also believed that HA-CD44 pathway is correlated to reproductive pathology, for instance, the CD44 receptor has been implicated in the development and metastasis of ovarian tumors and uterine carcinomas in women (Qu et al. 2001, Stokes et al. 2002). Whereas reproductive neoplasms are rare in mares, chronic inflammatory degenerative changes are often identified in old multiparous mares. Several pieces of evidence link CD44 to fibroblast adhesion, migration, and fibrosis development, for instance, CD44 receptors are frequently expressed in newly formed fibrotic tissue moreover. Furthermore, anti-CD44 may inhibit fibroblast invasion of a fibrin matrix, indicating interruption of the CD44 role can reduce or prevent fibrotic reaction (Svee et al. 1996).

Endometrial fibrosis is a chronic disease that can arise from intrauterine infections, irritant topical treatments, or multiple pregnancies; fibrosis relates to infertility through impairing glandular and epithelial function, resulting in embryonic mortality during the first trimester of pregnancy (McKinnon 1988).

HA-CD44 is reported to be involved in pathological conditions that may cause reproductive failure in mares due to its role in the development of inflammation and fibrosis (Rodriguez et al. 2011).

In agreement with our findings, there was no expression CD44 observed in the follicular phase of the menstrual cycle in the normal women endometrium (Yaegashi et al. 1995, Poncelet et al. 2002). Furthermore, it has been documented that the increased expression and significant endometrial shedding of CD44 may have a role in the pathogenesis of endometriosis in women (Griffith et al. 2010, Pazhohan et al. 2018).

Our results may refer to the proinflammatory function of CD44 which facilitates leukocyte extravasation and chemotaxes, induces inflammatory gene expression in leukocyte cells and contributes to matrix remodeling (Knudson, et al. 1993, DeGrendele et al. 1996, Puré and Cuff 2001). Additionally, evidence that CD44 plays a key role in many inflammatory diseases was obtained by studies wherein anti-CD44 reduced inflammation in murine models for inflammatory bowel disease, collagen- and proteoglycan-induced arthritis (Zeidler et al. 1995), skin inflammation (Camp et al. 1993), autoimmune encephalomyelitis (Brocke et al. 1999).

Endometrial receptivity is widely investigated in women. It could be defined as the ability of uterine mucosa to support successful embryonic implantation. Endometrial receptivity is mediated by molecular and genetic elements, like cytokines, adhesion molecules (integrins), growth factors (EGF, TGF-β, FGF), transcription factors (Hox), and ovarian hormones (Achache and Revel 2006).

The gene ITGB3 encodes for a cell-surface adhesion protein, which belongs to the integrin family that is cation-dependent transmembrane receptors composed of α and β subunits (Hynes 1992). ITGB3 is present in two combined forms either with the alpha IIb chain (Integrin alpha-IIb / beta-3 (αIIbβ3) or with integrin alpha-V / beta-3 (αvβ3). It is a cell adhesion coreceptors of the immunoglobulin that engage in cell adhesion and cell-surface mediated signaling (Hynes 1992, Brakebusch and Fassler 2003).

Integrins are related to various physiological processes including reproductive biology (Bowen and Hunt 2008) for instance, integrins are considered necessary for fertilization, embryo implantation and placental growth (Lessey et al. 1992, Damsky et al. 1993). Integrin expression

patterns are reported in pregnant and cycling cows (Kimmins and MacLaren 1999, McIntyre et al. 2002), sows (Bowen et al. 1996), women (Lessey et al. 1996) and mice (Illera et al. 2000).

In equine, the transcriptomic analysis of uterine biopsies on day 16 of pregnancy reveals overrepresentation of the integrin signaling pathway. It is hypothesized that the endometrial integrin αvβ3 interacts with conceptus-derived fibrinogen, contributing significantly in the cessation of conceptus mobility and fixation at days 15–16 of pregnancy (Klein 2015).

Integrin αvβ3 is documented to appear abruptly in the luminal and glandular endometrial epithelium at the putative implantation time in women coinciding with the increase in progesterone during the implantation window (Lessey 1998), while the high levels of estrogen (E2) prevent the implantation of the embryo. E2 is reported to decrease the expression pattern of integrin αvβ3 in women of with unexplained infertility (Lessey et al. 1995). E2 and progesterone (P4) tend to regulate the patterns of expression of integrins. Elevated blood E2 levels have been correlated with a substantial decrease in the level of integrin αvβ3 expression in human uterine biopsy samples (Chen et al. 2016), which agrees with our findings regarding the low expression of ITGB3 in RP mares due to the high levels of E2 at the sampling time in estrus. Interestingly, the SNP mares showed high uterine expression of ITGB3 regardless of the high E2 concentrations.

On the other hand, it was shown that ITGB3 mRNA expression in the normal bovine endometrial epithelial cells was downregulated following the inoculation of bacterial lipopolysaccharides (Guowu et al. 2019). However, this result was obtained from an in vitro culture of endome¬trial epithelial cells without any information about the reproductive hormone levels in the uterus before cultivation.

The high uterine expression of ITGB3 in SNP mares despite the high E2 concentrations in estrus can be attributed to the potential role of ITGB3 in the regulation of macrophage- related inflammatory reactions through activation of NF-κB. It is believed that the activation of integrin αVβ3 sustains the chronic inflammatory reactions under pathological conditions (Antonov et al. 2011). It is assumed that Integrin αVβ3 plays an important role in the onset and/or progression of a variety of human inflammatory diseases, such as osteoporosis and rheumatoid arthritis (Antonov et al. 2004, Wilder 2002).

In the present study, we assume that the SNP mares may have shown a certain degree of chronic uterine inflammation resulting in a high expression of ITGB3.

EGF is a member of the epidermal growth factor superfamily. It stimulates cell growth, proliferation and differentiation in a wide range of tissues through binding to its receptor (EGFR) on the cell surface. Such binding triggers a signal transduction cascade resulting in a wide range of biological changes within the cell, like increasing the intracellular calcium levels, enhancing glycolysis and protein synthesis (Fallon et al. 1984).

The uterine mRNA expression of EGF gene is associated with elevated serum estradiol concentrations during the follicular phase of menstrual cycle in women and rhesus monkeys (Imai et al. 1995, Ace and Okulicz 1995). In contrary, the mRNA expression of EGF in equine uterus has a different pattern of hormonal regulation compared to the previously mentioned species. In ovariectomized, prepubertal, and anestrous mares that were not under the influence of gonadal steroid hormones there was no evidence of EGF gene expression. Similarly, a tiny levels of EGF gene expression was found in the normal mares' uterus during estrus under the influence of estrogen. Moreover, estrogen treatment was unable to upregulate EGF gene expression in the endometrium of anestrous and ovariectomized mares. This indicates that the uterine mRNA expression of EGF in equine is not estrogen-dependent, but progesterone-dependent (Gerstenberg et al. 1999).

Endometrial EGF in mares appears to play a significant role during the formation of an epitheliochorial placenta, and glandular histotrophic development (Gerstenberg et al. 1999). Klein (2015) also reported that on day 16 of pregnancy, equine endometrium exhibits a high EGF mRNA expression.

In healthy cows, the uterine EGF protein levels are recorded to be at the maximum on days 2–4 and 13–14 and at the minimum on day 7 of the estrous cycle, while in repeat breeder cows the 2 peaks in the endometrial EGF profile are absent (Katagiri et al. 2004, Katagiri et al. 2006). The EGF concentrations in the uterus of fertile cattle display a cyclic shift during the estrous cycle. The loss of a cyclic EGF profile during the estrous cycle may reduce fertility in dairy cows (Katagiri et al. 2013).

In our study, RP mares showed low levels of EGF expression during estrus which agrees with the findings of Gerstenberg et al. (1999). Surprisingly, on the other hand, the SNP mares

exhibited high expression of EGF irrespective of the high level of estrogen at the time of sampling. We assume that an abnormal increase of EGF expression in SNP mares may increase its susceptibility to PBIE due to the estrogen-like effects of EGF which is known to be an important mediator of estrogen action in the reproductive tract (Nelson et al. 1991). One of these effects is that estradiol can promote the pro-inflammatory response of the uterus via regulating the expression of various cytokines (Tibbetts et al. 1999).

Overall, our findings in RP mares regarding the low endometrial expression of ITGB3 and EGF in healthy mares during the estrus phase confirm the previous equine studies. on the other hand, the abnormally increased expression of both genes in SNP mares may indicate their abnormal response to reproductive hormones which may increase the susceptibility to develop PBIE. More studies are required to explain the influence of E2 and P4 hormones on the expression of these genes during the estrous phase in fertile and infertile mares, in particular with evidence that infertile women with endometriosis have partial resistance to reproductive hormones during the menstrual cycle (Burney et al. 2007, Bulun 2010).
The IL23A gene encodes for a protein forming the interleukin IL-23. IL23 is a heterodimeric cytokine essential for the inflammatory response against infection through upregulating the matrix metalloprotease (MMP9) and enhancing angiogenesis. IL-23 modulates its impact on both innate and adaptive immune systems that express IL-23 receptors such as T helper 17 (Th17) cells that in turn release IL-17. Proinflammatory cytokines enhance T cell priming and trigger the output of other proinflammatory molecules such as IL-1, IL-6, TNF-alpha, NOS-2 and chemokines resulting in acute inflammation in peripheral tissues (Memari et al. 2015). IL-23 and IL-17 have been associated with the pathogenesis of some autoimmune disorders such as inflammatory bowel disease and psoriatic arthritis in humans (Cătană et al. 2015, Suzuki et al. 2014).

Recently it has been reported that women with unexplained repeated implantation failure have significantly higher expressions of IL23A, IL17, and IL6 in their uterine biopsies compared to healthy women (Amjadi et al. 2020). Such results are in agreement with our findings, which showed that the SNP mares showed a higher expression of IL23A higher than RP mares. It is assumed that IL23A can trigger the release of the proinflammatory IL6 cytokine, which is believed to be essential for the control of post-breeding inflammatory reactions in mares (Woodward et al. 2013b).

4.4 Lower expressed genes in susceptible mares that become pregnant compared to susceptible mares that did not become pregnant

SP mares displayed lower expression of some genes compared to SNP mares, these DEGs are significantly enriched for cellular component organization and biogenesis (degradation of the extracellular matrix, focal adhesion), regulation of biological (negative regulation of proteolysis) and developmental processes (cell junction assembly). A disintegrin and metalloprotease with a thrombospondin motif5 (ADAMTS 5), Collagen type V alpha 2 (COL5A2), Collagen type IV alpha 3 (COL4A3) and Fibronectin (FN1) genes were higher expressed in SNP mares than in SP mares and such genes are related to extracellular matrix (ECM) degradation, tissue adhesions and fibrosis.

ECM is a non-cellular component found in all organs and tissues providing physical support to the cells. ECM is a highly dynamic structure that is frequently remodeled by enzymatic or non-enzymatic means to maintain homeostasis of tissues (Järveläinen et al. 2009, Schmidt and Friedl 2010). Many proteases are implicated in the proteolytic degradation of ECM proteins, the most common of which are members of the matrix metalloproteinases (MMP), ADAMTS and tissue inhibitor of metalloproteinases (TIMPs) (Reiss and Saftig 2009, Egeblad et al. 2010). In the uterus, proteolytic degradation regulates the ECM proteins. Any imbalance of the ECM degradation process can lead to implantation abnormalities and infertility (Skinner et al. 1999).

The family of ADAMTS genes consists of 19 proteases with a wide range of functions in different tissues, like ECM remodeling, angiogenesis, fibrosis and coagulation. Many functions are reported for ADAMTS proteases under various physiological and pathological conditions, such as implantation, embryogenesis, inflammation, tissue degradation and repair (Shiomi et al. 2010, Nandadasa et al. 2014).

Our results demonstrated lower expression of ECM organizing genes ADAMTS 5, collagen COL4A3 and COL5A2 in SP mares compared to SNP mares. Similar results from bovine studies have shown that endometrial expressions of ECM-related genes (ADAMTS 5 and COL1A2, COL3A1, COL7A1 and COL3A3) are downregulated in cows with a high endometrial receptivity and healthy cows compared to cows with a low endometrial receptivity (Scolari et al. 2016). ADAMTS5 is also linked to men's infertility, as sperm counts, and motility has been reported to show a negative correlation with levels of ADAMTS5 protein in semen (Aydos et al. 2016).

Collagen is the key element of the ECM and plays a crucial role in wound healing. However, excessive synthesis of collagen contributes to organ fibrosis (Dupuy et al. 2019, Desogere et al. 2019). COL5A2 is known as a regulatory fibril-forming collagen, its expression is significantly up-regulated in conjunction with fibrosis in the renal epithelial cell lines and rat liver fibrosis (Zhang et al. 2015).
Our results show high mRNA expression of COL5A2 and COL4A3 in the endometrium of SNP compared to SP mares. Recently, it has been shown that the expression of COL5A2 is up regulated and linked with the degree of intrauterine adhesions (Chen et al. 2020). In accordance with our results, an abundance of collagen genes including COL4A3 was upregulated in low-fertility cow heifers in a study of Killeen et al. (2016). On the other hand, a lower expression of type IV collagen is observed in the decidua of spontaneously aborting women (Aplin 1996, Iwahashi et al. 1996).

FBN1 and FN1 genes are encoded for extracellular matrix glycoproteins. FN1 plays a crucial role in cell adhesion, growth, migration, and differentiation, and is important for processes including wound healing and embryonic development (Pankov et al. 2002). A high expression fibronectin has been associated with many pathologies, including cancer and fibrosis (Williams et al. 2008).

Our study shows that SNP mares expressed elevated levels of FN1. Similarly, the endometrial expression of FN1 has been reported to be significantly higher in non-pregnant compared to pregnant canine uteri (Graubner et al. 2018). FN1 is also associated with women's infertility. FN1 is reported to be the most up-regulated gene in women with uterine leiomyoma (Litovkin et al 2008). Moreover, the level of mRNA expression of the FN1 gene is higher in women with endometriosis compared to healthy women (Deraya et al. 2020). In one study of Bauersachs et al. (2005) mRNA expression of FN1 and COL5A2 was upregulated in the bovine uterus during the estrus. However, the authors of this study did not relate the expression of these genes to pregnancy results.

Collagen IV and FN1 deposition outside the basement membrane is observed in the equine uterus in the case of glandular fibrosis (Walter et al. 2001). Hence, we believe that the SNP mares may develop a more distinct endometrial fibrosis as they expressed higher levels of ADAMTS 5, COL5A2, COL4A3 and FN1 than SP mare which may be an explanation for pregnancy failure in this mares.

4.5 Genes of higher expression in susceptible mares that become pregnant compared to susceptible mares that did not become pregnant

The present study identified some genes higher expressed in SP mares compared to SNP mares. These genes are associated with cellular response to growth factor stimulus, signaling, cell proliferation and developmental processes involved in reproduction. Fibroblast growth factor 9 (FGF9), Growth differentiation factor 10 (GDF10), kinase insert domain receptor (KDR) and C-X-C motif chemokine ligand (CXCL) 16 genes were higher expressed in SP mares compared to SNP mares. These genes are related to uterine cell proliferation, differentiation, and angiogenesis.

The FGF9 gene encodes for a protein belonging to the FGF family, which induce various cellular functions such as migration, proliferation and differentiation (Yun et al. 2010, Itoh et al. 2011). It affects a wide range of physiological and pathological processes, such as embryonic development, angiogenesis and tumorigenesis (Beenken and Mohammadi 2009). The biological function of FGFs is regulated through binding with transmembrane tyrosine kinase receptors. FGFR is encoded by four distinct genes (FGFR1–FGFR4) (Eswarakumar et al. 2005). FGF9 binds with high affinity to FGFR2 and FGFR3 (Ornitz et al. 1996).

FGF9 functions as paracrine and/or autocrine mediators of epithelial-stromal interactions which trigger luminal epithelium proliferation in the uterus (Fujimoto et al. 1997, Filant et al. 2014). In the uterus of fertile women, FGF9 is expressed at high levels, particularly during the late proliferative phase, which correlates with the rise of estradiol and the time of uterine endometrial proliferation (Tsai et al. 2002). Furthermore, FGF9 is reported to be a major element required to establish an optimal microenvironment for successful implantation and pregnancy in mares, women, mice and sows (Merkl et al. 2010, Tsai et al. 2002, Šućurović et al. 2017, Ostrup et al. 2010).

The equine conceptus starts to secrete substantial amounts of estrogens on day 10 of pregnancy (Choi et al. 1997), which are expected to have many impacts on early pregnancy including stimulation of early conceptual migration, alterations in uterine tonicity and blood flow, and endometrial secretory activity, which is important for the nutrition of the preimplantation concept (Stout and Allen 2001). FGF9 is an important mediator of estrogen signaling in the equine uterus. It is reported to be upregulated during estrus and on day 12 of pregnancy in the normal equine endometrium (Gebhardt et al. 2012, Merkl et al. 2010).

In addition, the mRNA expression of FGF9 is reported to be higher in the uterus of pregnant sows and women compared to the non-pregnant individuals (Ostrup et al. 2010, Tsai et al. 2002).

The GDF10 is another gene encodes for growth factor protein found to be higher expressed in SP relative to the SNP. GDFs are members of the transforming growth factor-b (TGF-b) superfamily that participate in many cellular functions and biological processes such as cell proliferation, differentiation and remodeling (Whitman 1998).

Earlier research reported the role of GDF genes in the regulation of prenatal uterine development (Tong et al. 2004). The members of the GDF family have also been shown to play a critical role in the regulation of oocyte development (Gui and Joyce 2005). GDF10 mRNA expression is reported to be high in the pregnant endometrium of cattle (Mamo et al. 2012). Furthermore, it has been reported that the endometrium expresses elevated levels of GDF10 on day 16 of pregnancy in the mares (Klein 2015).

In agreement with Klein (2015) and Gebhardt et al. (2012), our results showed higher expressions of FGF9 and GDF10 in SP mares compared to SNP mares. We believe that susceptible mares with high FGF9 and GDF10 profiles may become pregnant if the accumulated intrauterine fluid is properly handled within breeding.

Vascular endothelial growth factor receptor 2 (VEGFR2) or kinase insert domain receptor (KDR) gene encodes for a tyrosine-protein that functions as a cell-surface receptor for vascular endothelial growth factor (VEGF) that plays an important role in the regulating of angiogenesis, vascular production, vascular permeability and embryonic hematopoiesis. Besides, it induces endothelial cells proliferation, survival, migration, and differentiation (Gerber et al. 1998, Jia et al. 2004). VEGF regulates normal uterine vascularity through its action on endothelial cells of blood vessels via two membrane receptors: VEGFR1 and KDR (Sağsöz and Saruhan, 2011). Signaling via KDR is suggested to be responsible for this VEGF activity rather than through VEGFR1 (Ferrara et al. 2003). VEGF ligand and its receptors are present in the endometrium's luminal epithelium, glandular epithelium, stroma, and blood vessels with variable expression during the cycle, indicating reliance on ovarian steroids (Tasaki et al. 2010).

Cyclic morphological changes in the endometrium are accompanied by a significant remodeling of the vasculature to create a receptive environment (Torry et al. 2007). Such vascular remodeling depends on hormonally regulated angiogenesis (Walter et al. 2010). Angiogenesis is a critical aspect of normal implantation/placentation at the early stages of pregnancy. Many studies have been focused on the existence of the signals responsible for triggering and regulating endometrial angiogenesis (Girling and Rogers 2009, Herve' et al. 2006). They have investigated the levels of VEGF and its receptors in the pregnant and non-pregnant uterus and found cycle-dependent expressions in rabbits (Das et al. 1997), pig (Winther et al. 1999), human (Moller et al. 2001), cows (Sağsöz and Saruhan, 2011) and sheep (Tremaine and Fouladi-Nashta 2018).

The upregulation of KDR expression is associated with increased levels of estrogens and VEGF (Herve' et al. 2006). In addition, Rockwell et al. (2002) found that immunosuppression of VEGF decreased the estrogen-induced uterine edema and blocked implantation.

In mares, the pattern of immunohistochemistry localization of KDR differs between pregnant and cyclic mares. KDR staining is low in the luminal endometrial cells of cyclic mares during estrus (Allen et al. 2007). KDR expression was highest in pregnant mares on day 21. This may indicate the role of the embryo-derived estrogens at the time of implantation, suggesting the involvement of VEGF and KDR pathways in stimulating the production of maternal and fetal vascular networks for the exchange of gases, nutrients and waste products during pregnancy (Silva et al. 2011).

However, an earlier study of Allen et al. (2007) showed a low level of KDR expression in equine endometrium during estrus, our study demonstrated that SP mares have a higher mRNA expression of KDR than SNP mares during estrus, this discrepancy may be attributable to the use of different techniques for the assessment of KDR expression in the equine uterus as Allen et al. (2007) used immunohistochemistry to localize the KDR protein while we used transcriptomics to detect the KDR gene expression, we assume that the PBIE susceptible mares with high KDR profiles can become pregnant if the intrauterine fluid is controlled by using ecbolic drugs to remove fluid accumulations after AI.

Chemokines are a special type of cytokines that are specifically recognized to have a role in leukocyte trafficking, CXCL16 is a member of the chemokine family, and CXC chemokine

receptor (CXCR6) is its main receptor (Matloubian et al. 2000). Emerging evidence has shown that CXCL16/CXCR6 is expressed at the maternal-fetal interface by cell types including trophoblast cells, decidual stromal cells, and decidual immune cells (e.g. monocytes and T cells). CXCL16 plays a crucial role in establishing a successful pregnancy via many molecular interactions at the maternal-fetal interface (Shi et al. 2019).

In equine, the uterine mRNA expression of CXCL16 is reported to be at the highest level during the follicular phase of the estrous cycle, which affirms the assumption that the cytokine CXCL16 plays an important role in the uterine clearance after AI (Gebhardt et al. 2012). Cytokines are triggered by the entrance of sperm and microorganisms (Troedsson et al. 2001), which contribute to the influx of polymorphonuclear neutrophils into the uterine lumen (Troedsson 1999).

The existence of an embryo in the uterus mediates the immunomodulatory activity of the uterus, which is further emphasized by the up-regulation of several chemokines (including CXCL16) necessary as regulators of maternal receptivity and embryo implantation (Sharkey 1998, Tartakovsky and Ben-Yair 1991). In addition to promoting cell proliferation and the invasion of trophoblasts (Simon et al. 1998, Zheng et al. 2007), CXCL16 / CXCR6 chemokine receptor-axis is stated to be up-regulated in the pregnant canine uterus to induce proliferation and to modulate the immune system in preparation for implantation and placenta (Graubner et al. 2017).

Human studies show that CXCL16 is impaired at the human maternal-fetal interface in patients with recurrent pregnancy loss (Huang et al. 2008, Fan et al. 2019). It is observed that CXCL16 encourages the proliferation and autocrine invasiveness of primary-cultivated trophoblasts (Huang et al. 2006). In addition, CXCL16 is known to be an initiator of molecular crosstalk that stimulates the decidualization of endometrial cells, indicating that CXCL16/CXCR6 chemokine plays a significant role in the development and progression of human placenta (Mei et al. 2019).

The present study shows higher mRNA expression of CXCL16 in the SP mares uterus compared to the SNP mares, which may indicate the role of CXCL16 in early implantation and pregnancy establishment.

4.6 Limitations of the study

Mares in the present study were classified into mares that did become pregnant or not based on the result of pregnancy checks after the artificial insemination using semen of different stallions. We cannot rule out the possibility of pregnancy being affected by the fertility of the individual stallions, however, we included only mares that were inseminated with the semen of fertile stallions. Furthermore, the hierarchical cluster analysis of the DEGs showed only partial separation of the different mare groups for both SNP-RP and SP-SNP comparisons. This could be due to some weakness in the classification of the mares. The animals and the breeding management of selected mares of the different groups of mares were similar, but not completely the same. In addition to the number of mares included in our study was relatively low.

4.7 Conclusion

In conclusion, the present study shows substantial differences in the uterine transcriptome profiles between susceptible mares that did not become pregnant in comparison to resistant mares that became pregnant in estrus even before the breeding challenge. We have identified a number of DEGs as possible molecular biomarkers for the susceptibility to PBIE, such transcripts of genes associated with steroid hormones metabolism, neutrophils chemotaxes, development, and maintenance of uterine inflammation. Furthermore, we have demonstrated that even among mares that tend to accumulate abnormal intrauterine fluids, there were significant differences in the uterine transcriptome profiles that may be related to the mare's ability to become pregnant. The DEGs between susceptible mares that became pregnant or not were assumed to be useful indicators for the fertility of susceptible mares if they receive proper breeding management. These DEGs are related to ECM degradation, tissue adhesions fibrosis, uterine cell proliferation, differentiation, and angiogenesis. In order to verify our findings regarding the prediction of PBIE susceptibility and the overall fertility of broodmares, further studies are needed with a focus on the expression of specific genes in larger populations of susceptible and resistant mares to PBIE.

5. References

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Curriculum Vitae

First name Family name	Amr Salah Mohamed Elshalofy
Date of birth	25/09/1990
Place of birth	Damietta, Egypt
Nationality	Egyptian
09/1994 – 05/2004	**Primary school** (Mubarak school, Damietta, Egypt)
09/2004 – 05/2007	**High school** (Khaled Ben El Waleed Secondary school, Damietta, Egypt)
09/2007 – 05/2012	**University** (Faculty of Veterinary medicine, Cairo University, Giza, Egypt)
05 / 2012	**Veterinary medicine degree examination** (Faculty of Veterinary medicine, Cairo University, Giza, Egypt)
01/2016	**Master degree of Theriogenology** (Faculty of Veterinary Medicine, Cairo University, Giza, Egypt)
09/2018 – 08/2020	**Preparation of the thesis** Under the supervision of Prof. Dr. med. vet. Heinrich Bollwein PD Dr. rer. nat. Stefan Bauersachs at the Department for Farm Animals of the Vetsuisse Faculty University of Zurich Director Prof. Dr. med. vet. Heinrich Bollwein

Signature: